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尋找新型流感病毒抑制劑及研究其藥物機轉
Screening novel influenza virus inhibitors and anti-Flu mechanism of these drugs

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（中文）

尋找新型流感病毒抑制劑及研究其藥物機轉

（英文）

Screening novel influenza virus inhibitors and anti-Flu mechanism of these drugs

經本委員會審定通過，特此證明。

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六年的博士班生涯終於到了尾聲。大太陽底下奮力鳴叫的知了催促著夏天的腳步。

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無數的夜晚，結束了醫院工作後，回到家仍打起精神埋首苦讀的日子將暫告一個段落。醫學進步日新月異，學術研究永遠有新穎的題材等著人們探索...前方又是嶄新的航道，在師長、家人、太太、朋友...的陪伴下，繼續航向充滿希望的旅程。

目錄

中文摘要

流感病毒(Influenza virus)的基因,具有多樣性的抗原漂移(antigenic drift）和抗原轉變（antigenic shift）的能力，導致高致病性且具高傳染性的流感病毒暴露在人類社會，造成大流行。每年流感病毒的疫情，導致全球300萬到500萬個嚴重病例，並造成其中50萬人死亡。由於疫情的迫切性，市面上有四種美國食品藥品管理局批准的抗流感病毒藥物：amantadine、rimantadine、oseltamivir 和 zanamivir。於2005-2006年流感季節之後,發現A型流感病毒已對 amantadine 產生了九成以上的抗藥性。因此，只有神經胺酸酶抑製劑（oseltamivir 和 zanamivir）對目前的流感病毒仍然有效。2009年4月，H1N1流感大流行在世界各地蔓延，全球 oseltamivir 產生抗藥性的病例越來越多，再次顯示抗病毒藥物的大量使用讓抗藥性的病毒株快速增加。由於這些原因，研發對抗流感病毒藥物是目前迫切的課題。快速並準確檢測流感病毒的方式，是成功發展抗病毒藥物和疫苗研製的關鍵。傳統使用病毒斑分析(plaque assay)和細胞50%生長抑制所需的藥物濃度(GI50)，仍是研究新型流感病毒藥物最常使用的方式，然而這種方式相當費時費力。在這項研究中，我們使用偵

測流感病毒的冷光報告系統(influenza-specific luciferase reporter assay)，快速且客觀地定量病毒、病毒中和試驗、藥物篩選試驗和抗病毒基因的影響，以證明病毒誘導的冷光細胞株之效用。本研究測試了300種中國傳統治療呼吸道疾病的藥物，利用受到流感病毒感染的A549細胞株，加入中藥後，利用流感特異性冷光報告系統分析。在這些測試的傳統中藥中，我們發現虎杖 (Polygonum cuspidatum) 和它的活性化合物白藜蘆醇 (resveratrol) 和大黃素 (emodin) 可以減少A549細胞中流感病毒的複製。受到抑制的病毒，包括2009到2011年從台灣分離的臨床流感病毒檢體和實驗室的標準A型流感病毒株A/WSN/33 (H1N1)。虎杖、白藜蘆醇和大黃素也都會抑制血球凝集素和神經胺酸酶的表現，而且會經由TLR9增加ß干擾素的表現。此外，當受到流感病毒感染的A549細胞加上抗β干擾素抗體和TLR9抑制劑之後，β干擾素的產生或白藜蘆醇的抗病毒活性也跟著降低。顯示β干擾素和白藜蘆醇對於抑制H1N1流感病毒有很好的協同作用。這個潛在的抗病毒機轉，包括直接抑制病毒的複製和刺激宿主對抗病毒的免疫反應。此一現象在單一抗病毒分子還未被發表過。總結是，我們的研究發現使用虎杖、白藜蘆醇或大黃素抑制流感病毒的複製是經由TLR9誘導β干擾素的產生。

關鍵詞：流感病毒、抗藥性、虎杖、白藜蘆醇、β干擾素、TLR9

Abstract

Influenza virus cause 3-5 million cases of severe illness and up to 500000 deaths worldwide per year. Influenza viruses are highly diverse with antigenic drift and shift, which could lead to emergence of viruses that have been exposed to human population, leading to pandemic outbreaks. Because of the importance of influenza virus infection, there have been major efforts in the development of effective antivirals for the last several decadesThere are now four U.S. FDA-approved drugs on the market; amantadine, rimantadine, oseltamivir, and zanamivirin the 2005/2006 flu season, 92.3% of influenza A viruses analyzed from 26 states in the U.S. contained point mutations that conferred amantadine resistance. Therefore, only the NA inhibitors remain effective against the currently circulating viruses. In 2009, a H1N1 pandemic occurred in April and spread across the world. More and more oseltamivir-resistant viruses strains spread quickly worldwide, indicating again that antiviral resistance arises quickly with drug use. For these reasons, identification of new anti-viral compounds from natural products is important for development of therapeutic agents against newly appeared influenza virus. Accurate, specific, and rapid detection and quantification of influenza virus are critical for successful development of diagnostic tests, antiviral drugs and vaccines. Assays that are based on

virus-induced cytopathic effects such as the plaque assay study and the 50% inhibition of cell growth (GI50) are still commonly used to quantify influenza virus, but such assays are time-consuming and labor- intensive. In this study, we developed a detection system for influenza A virus using a luciferase reporter gene system and demonstrate the utility of these cell lines in objective, rapid, and quantitative assays for virus quantification, virus neutralization assays, drug screening assays and cytokines response after treating influenza A virus. Here we tested the effects of 300 traditional Chinese medicines on the replication of various influenza virus strains in a lung cell line, A549, using an influenza-specific luciferase reporter assay. Of the traditional medicines tested, Polygonum cuspidatum (PC) and its active components, resveratrol and emodin, were found to attenuate influenza viral replication in A549 cells. Furthermore, they preferentially inhibited the replication of influenza A virus, including clinical strains isolated in 2009 and 2011 in Taiwan and the laboratory strain A/WSN/33 (H1N1). In addition to inhibiting the expression of hemagglutinin and neuraminidase, PC, emodin, and resveratrol also increased the expression of interferon beta (IFN-β) through Toll-like receptor 9 (TLR9). Moreover, the anti-viral activity of IFN-β or resveratrol was reduced when the A549 cells were treated with neutralizing anti-IFN-β antibodies or a TLR9 inhibitor,

suggesting that IFN-β likely acts synergistically with resveratrol to inhibit H1N1 replication. This potential antiviral mechanism, involving direct inhibition of virus replication and simultaneous activation of the host immune response, has not been previously described for a single antiviral molecule. In conclusion, our data support the use of PC, resveratrol or emodin for inhibiting influenza virus replication directly and via TLR-9–induced IFN-β production.

Keywords: Influenza virus、influenza-specific luciferase reporter assay、IFN-β、TLR9、PC,、resveratrol、emodin

前言

流感病毒概略

流感病毒是一種造成人類及動物感染流行性感冒的分段單股負鏈 RNA 病毒 (segmented negative strand RNA virus)。在病毒分類學上，流感病毒屬於正黏液病毒科中的正粘病毒屬（Orthomyxoviridae）[1,2]。此單鏈 RNA 含包膜的病毒具有螺旋對稱，直徑約 80-120nm。RNA 被緊密地與核蛋白相聯，以形成螺旋結構。

病毒基因組是分段的，A 型與 B 型流感病毒為 8 個 RNA 片段，C 型流感病毒為 7 個 RNA 片段。每一段 RNA 都會形成特殊的多醣體，並有其特殊的功能[3,4]。第一段到第三段 RNA 分別形成 PB2、PB1 和 PA，三者有轉錄酶 (transcriptase) 的效果：分別帶帽 (cap binding) 延長 (elongation) 和蛋白酶活性 (protease activity)，而 PA、PB、PB2 三種多醣體組合形成 RNA 依賴的 RNA 聚合酶 (RNA-dependent RNA polymerase)，將 RNA 作為模板來合成新的 RNA 鏈[5]。第四段和第六段 RNA 最為重要，即血球凝集素 (Hemagglutinin) 和神經胺酸酶 (Neuraminidases) 分別負責病毒進入和釋出呼吸道上皮細胞。A 型流感

病毒依表面抗原血球凝集素（HA 抗原）及神經胺酸酶（NA 抗原）的不同，另可分為許多亞型。其中，H 抗原亞型共有 18 種，為 H1-H18；N 抗原亞型共有 11 種，為 N1-N11。B 型及 C 型流感病毒則不區分亞型。第五段和第七段形成結構蛋白和 M1 蛋白 (Matrix protein) 和 RNA 聯結維持單股螺旋構造。第七段 RNA 還會形成另一結構蛋白 M2 protein，為氫離子通道。第八段 RNA 形成 NS1、NS2 等非結構蛋白形成，胞漿和核仁。

病毒體內有兩種醣殼蛋白(core proteins)包括:核蛋白（nucleoprotein, 簡稱 NP）和基質蛋白（matrix protein, M1）。這兩種醣殼蛋白提供了流感病毒的分類型特異性抗原，流感病毒的兩種醣殼蛋白（M1 及 NP）所產生的 CF 抗體，可以將流感病毒分為 A、B、C 三型。基質蛋白包圍核蛋白，佔流感病毒粒子質量的 35-45%[6]。病毒表面有兩種醣蛋白，血球凝集素(Hemagglutinin, 簡稱 HA)和神經氨酸酶(Neuraminidase, 簡稱 NA); 血球凝集素在病毒表面形成棒狀突起，並且由 3 個亞基 HA1，HA2 和 HA3 所構成。流感病毒藉著血球凝集素與宿主呼吸道細胞表面的醣分子唾液酸層 (sialic acid) 接受器結合，結合後宿主細胞吞入病毒，病毒 RNA 入侵宿主呼吸道上皮細胞核，即利用宿主細胞核大量複製病毒。複製好的 RNA 與蛋白於細胞表面出芽而形成新的病毒，此表面的

神經氨酸酶就會幫病毒分解呼吸道上皮細胞表面的唾液酸，新病毒離開此呼吸道上皮細胞後便自由地擴散，進入其他未受感染的細胞繼續複製和破壞[7]。

流感病毒的突變

血球凝集素的 18 種亞型之中，人類常見：H1、H2、H3、H5、H7 和 H9。豬隻中常見 H1 和 H3，馬常見 H3 和 H7，而鳥則會攜帶 H1 到 H16 等所有的流感病毒原型株從北方的西伯利亞往南飛。在神經胺酸酶的 11 種亞型中，人類常見 N1 和 N2，豬隻也是 N1 和 N2，馬是 N8 和 N9，而鳥則是攜帶所有 N1 到 N11 原型，南下到同時有人、豬、鳥的聚集地。禽流感病毒對於鳥類呼吸道上皮細胞的α2-3 連結之唾液酸有高的親合性。而人類的流感病毒，是對於人類呼吸道非纖毛細胞的α2-6 連結殘基有高度的親合性。所以禽流感病毒通常不會傳染給人，也不會造成人類之間的傳染。此為不同物種之間不會交互傳染的"傳染屏障"。禽流感病毒因不會辨識人類細胞而無法感染人類，亦不易造成人傳人感染[8]，但α2,3 或α2,6 醣類結合只是病毒結合特性，並非決定病毒是否會感染的唯一因素。豬的傳染屏障較弱，在豬隻的呼吸道上皮同時擁有

對人和禽流感病毒的唾液酸接受器(sialic receptor)，使得人流感和禽流感病毒在豬的體內進入基因交換。

分段的流感病毒基因如果產生點突變，或兩、三個胺基酸變異，即稱為「抗原漂移」(antigenic drift) [9]。抗原漂移大約兩、三年一次，造成地區小規模流行或大地區的流行。每次的流行，會使部分人類產生抗體，隨著有抗體的人越多，即失去病毒的傳染力[10]。另一種流感病毒變異的方式，是由兩種不同的病毒類型 (如 H1N1 和 H3N2) 混合，病毒間會互相交換自身的基因，進而創造出全新病毒。交換的基因如果發生在第四或第六段的全部 RNA 被取代，造成血球凝集素或神經胺酸酶的變異，導致病毒的表面出現變異，外圍的蛋白質變異，形成新的流感病毒，此即所謂的「抗原轉變」（antigenetic shift）[11,12]，此過程只能在已被感染的細胞中進行。由於此類變異時間迅速，一旦發生變異，大多數人類的免疫系統均來不及辨識此病毒，已變異的流感病毒即於人群中大量傳染，引發全國或全世界大流行。1957 年及 1968 年世界大流行的人類流感病毒，均含有類似禽類流感病毒的基因片斷。高致病性的 1997 年禽流感病毒 H5N1 及 2013 年 pandemic H7N9 病毒亦是禽流感病毒突變演化而成的重要案例。

流感病毒臨床現況

流感病毒中 A 型與 B 型流感病毒較容易於人類引起大規模的急性病毒性呼吸道疾病，產生明顯發燒、疲倦、頭痛、肌肉痠痛、流鼻涕、喉嚨痛以及咳嗽…等，甚至會造成死亡。病程通常為 2~ 7 天。流感爆發流行快速、散播範圍廣、併發症嚴重，尤其以病毒性及細菌性肺炎最為常見[13,14]。其中，目前所有已知的 A 型流感病毒型別中，H1N1、H2N2、H3N2 三型曾於人類的世界中，造成世界大流行（pandemics）及地區流行（epidemics）[15,16]；H5N1 禽流感暫時未有人與人之間互相傳染的實證[17,18]。

研究指出 2009 年墨西哥 H1N1 病毒，其中 6 段病毒基因是由之前在北美豬隻中流行的三種基因重組之豬流感病毒而來；另 2 段基因 HA 及 NA 基因則是從歐亞混種似禽類豬流感病毒而來[19]。2009 年 H1N1 較季節流感會引起較嚴重的肺部病變，且新型流感在感染動物的上呼吸道及下呼吸道複製較好，而季節流感病毒則侷限於在上呼吸道複製，這或許可以解釋為何新型流感導致部分較嚴重案例有病毒性肺炎症狀產生[20]。

台灣從 2009 年 5 月至 2011 年 4 月之間，收集了 1,335 株 H1N1 流感病毒，並且測試了神經胺酸酶的 H275Y 基因，此一突變將會對抗病

毒藥物「克流感」產生抗藥性。結果在其中 15 株(1.1%)H1N1 流感病毒中發現了 H275Y 基因的突變。於這兩年的收集時間當中，平均每個月收集到 H275Y 基因的突變株約 0%~2.88%。H275Y 基因無法維持穩定的突變，因此流感病毒內的 NA/HA 比例下降，進而導致流感病毒不穩定而死亡。這也是為何抗藥性 H275Y 流感病毒突變株無法造成全球散佈的原因之一 [21]。

21 世紀第一波全世界流感病毒 (H1N1) 大爆發發生於 2009 年 3 月的墨西哥和 2009 年 4 月的美國[22]。世界衛生組織把全球流感大流行警告級別提高到致第 5 級。WHO 於 2009 年 4 月 19 日至 12 月 26 日期間，153 國的流感陽性檢體中，66.6%為 H1N1 新型流感，26.6%為 A 型季節流感，6.5%為 B 型季節流感。2009 年度全球有 168 例克流感（oseltamivir）抗藥性病例，病毒均為 H275Y 突變，但對瑞樂莎（zanamivir）則無抗藥性。214 個國家在 2009 年至 2011 年經歷的 2~3 波的流感病毒侵襲，一共造成 18,449 位病人死亡。世界衛生組織於 2009 年 6 月發現：在神經胺酸酶的胺基酸 275 位置 histidine 被 tyrosine 取代，而產生克流感的抗藥性，於 2009 至 2011 年這兩年，全球累積了 605 位抗藥性個案[23]。在台灣，2008~2009 年季節性流感病毒(H1N1)的克流感抗藥性菌株比例

相當低，但是在 2008 年 9 月至 12 月之間病毒的抗藥性從 14.3%快速上升至 100%[24]。

2005~2006 年對「金剛胺」抗藥性的 H3N2 流感病毒和 2007~2009 年對「克流感」抗藥性的 H1N1 流感病毒的爆發，基因的重組(Reassortment)和突變，提供了流感病毒產生抗藥性的原因。但是 H275Y 突變之後的「克流感」抗藥性病毒株有可能會降低流感病毒的毒性(virulence) 和複製能力 (replication)，進而減少神經胺酸酶酵素的活性，和病毒在呼吸道的傳播能力[25]。

2013 年 2 月，中國發現了 H7N9 流感病毒的蹤跡，H7N9 流感病毒對於雞隻沒有高病原性，對於禽鳥類也僅造成些微症狀。但是卻造成黃浦江下游地區，包括：上海、安徽、浙江…等地造成人類死亡個案[26]。而在 2013 年 4 月 24 日台北市也分離出一株 H7N9 流感病毒。研究學者因 N9 和 N2 的基因結構類似而從 N2 之前的研究中發現：E119V、I222V、R2224K，R292K 和 N294S 的突變點均會降低「克流感」的藥效，也希望藉由以上研究來證實 H7N9 之中是否也含有這些克流感抗藥性的突變基因？研究發現此一台北的病毒株帶有 R292K 的抗藥性突變點[27]。美國的學者更進一步利用此一病毒株，從實驗動物中發現 NA-R292K 突變病毒株會強力抑制克流感和 peramivir。而 NA-I222K 突變的流感病毒對

小老鼠有最強的毒力，以及神經胺酸酶沒有變異與 R292K 變異之流感病毒株對小老鼠的毒性最弱。

抗流感病毒藥物

流感抗病毒藥劑是一種可以預防或治療流感病毒感染的藥品，藥物分為兩類：一為神經胺酸酶抑制劑 （neuraminidase inhibitor），另一為 M2 蛋白抑制劑（M2 ion channel blockers）[28,29]。2009 年 WHO 發佈的 H1N1 用藥指引中，強調有兩種神經胺酸酶抑制劑可以治療流感：一種是口服的克流感 (oseltamivir)，另一種是吸入型的瑞樂沙（Relenza, zanamivir），兩種都通過美國食品藥物管理局的認證，對於預防嚴重病情、減低住院機率與天數有明顯貢獻。適當使用克流感可明顯減少病人肺炎的風險，流感症狀出現後 48 小時內投藥療效最佳。另外，在美國還有一種 IV 形式的 Zanamivir，用於治療流感重症的病人。在日本還有一種長效型吸入的藥物 laninamivir。在南韓、日本、中國還有使用 IV 形式的 peramivir [30]。

抗病毒藥物是人類預防流感的最後一道防線，在尚未完成新藥開發以及抗藥性快速上升的此時，如何正確診斷流感病人以及正確使用流感病毒藥物是目前最重要的課題之一。

目前抗流感藥物的研發分為五大類[31,32]：

1. Adamantanamine derivatives：即 M2 氫離子通道阻斷劑。讓氫離子無法進入流感病毒，流感病毒無法因酸鹼不平衡而瓦解，病毒內的 RNA 也因而無法釋放於人體細胞核之中，而無法進行複製。
2. IMP dehydrogenase inhibitor：即病毒複製時，阻斷 GTP 能量的供給。
3. RNA polymerase inhibiter：RNA 聚合酶的抑制劑。
4. SiRNA：參與 RNA 干擾(RNAi)現象，以帶有專一性的方式調節基因的傳達，並參與 RNAi 相關的反應途徑。
5. Neuraminidase inhibitor：抑制神經胺酸酶，讓合成好的病毒無法離開宿主呼吸道細胞表面。

中國傳統中藥在抗流感藥物之發展

2009 年全球流感疫情爆發，病毒逐漸對克流感產生抗藥性病例增加，此時發展新的抗病毒藥物是刻不容緩的問題。由瑞士羅氏藥廠（Roche）所生產的抗流感病毒藥物「克流感」，含有自中藥材「八角」種子中所萃取分離的「莽草酸」（Shikimic acid）成分，再經包括化學反應等十

個步驟之生產過程，才製得主成分 Oseltamivir phosphate[33]。因此，本研究企圖從其它中藥材中，尋找未來開發抗流感病毒藥物的可能性。

本研究自「中國醫藥大學附屬化合庫」中，篩選 300 種對呼吸道感染有幫助之中藥材進行研究，發現「虎杖」對流感有最佳抑制效果。「虎杖」（Tiger Stick，Japan Fleece flower，Giant Knotweed ）[34]味微苦，別名：川七、班杖、酸杖、黃藥子、川筋龍、大蟲杖。原產地：台灣、江蘇、浙江、安徽、廣西、日本及韓國；台灣全島 2,400~3,800 公尺的山地都可看到它的踪跡。藥理作用：可對抗病毒和真菌、鬆弛支氣管平滑肌，鎮咳祛痰、降血壓及血脂、對外傷有明顯的止血作用、鎮痛、消炎、抗菌[35]例如： 綠膿桿菌, 金黃色葡萄球菌, 卡他球菌, 鏈球菌, 大腸桿菌…等。

流感病毒的偵測

本實驗之流感病毒辨識是經由細胞培養和使用免疫冷光特異性抗原細胞。確定流感病毒的培養方式曠日廢時，且經由抗原抗體反應確認的流感病毒變異性也太大。所以，近年來一種直接且快速抗原的流感病毒偵測系統已被開發[36,37]。但是新方法的敏感性和正確性仍然不足

[38,39]。另外，以核菌酸為系統 (nucleic acid based techniques)的 real time PCR 或 microarrays 也可以提供快速、專一、敏感的診斷系統[40-42]。

另一種偵測流感病毒的方式，是藉由報告基因 (reporter gene) 顯示病毒的促進因子 (promoter) 和病毒量的表達[43]。此套報告基因系統正被用於多種 RNA 病毒的追蹤[44-47]。流感病毒基因在宿主細胞中內的複製和轉錄，需要 Polymerase (包含：PA、PB 和 PB2 蛋白) 還有結構蛋白(nucleoprotein，簡稱 NP) 加到流感病毒的 RNA 之後，整個形成 viral ribonucleoprotein (vRNP) 複合體。在 5’和 3’端的 UTRs 位置所表現的順式 RNA 所複製或轉錄的保守結構[48-50]，可以當做是病毒的報告基因[51,52]。

對抗流感病毒的免疫機轉

在先天免疫階段 (innate immunity)，外來分子驅動 T 細胞的介入引導，病毒被感知成危險信號，進而啟動細胞因子 (cytokines) 和趨化因子 (chemokine) 的發展，而 TLR (toll like receptor) 最主要的工作就是識別外來的病毒、細菌、黴菌。TLR2, TLR4,和 TLR9 可以辨別外來的黴菌。然而，只有 TLR9 被證明在黴菌感染中，會先產生保護性的免疫反

應。反應初期的趨化因子，例如： IFN-γ、TNF-α、IL12、CCR2 ligands 都會加速 Th1 的發展。

哺乳動物體內三個重要偵測病毒的工具為：

1. Toll like receptor (TLR)，特別是 TRL3、TRL7、TRL8 和 TRL9 表現於核體內 (endosome)[53-55]。
2. RIG-I-like receptor (RLRs)，例如：RIG-I、MDA5 和 LGP2[56]。
3. NOD-like receptors (NLRs)，TLRs 和 NLRs 主要位於樹突細胞 (dendritic cells)、巨噬細胞 (marophages) 和 B 細胞。而 RLRs 的抗病毒效果則位於各類的細胞[57,58]。

病毒的外來刺激影響細胞下游的轉錄因子啟動，如：NF-κB 和 interferon-regulatory factors (IRF3 或 IRF7)，進行產生抗病毒的細胞因子，如：第一型干擾素 (IFN-α 和 IFN-β)。抗病毒的細胞因子更進一步誘導下游 IFN 訊號 (Jak/STAT) 路徑的傳遞，在細胞內啟動一些轉錄因子 STAT1 和 STAT2[59]，例如：干擾素刺激因子 interferon stimulated genes (ISGs)，例如：myxovirus-resistance protein (Mx) GTPase, RNA-dependent protein kinase (PKR), ribonuclease L (RNase L), Oligo-adenylate Synthetase (OAS) 和 interferon Stimulated Gene (ISG)[60-63]。這些抗病毒蛋白在人體啟動免疫反應後，直接或間接產生抗病毒的效果。流感病

毒被內吞作用 (endocytosis) 進入漿細胞樣樹突細胞 (plasmacytoid Dendritic Cells pDC)，病毒接觸溶小體 (lysosome)病毒的核酸 (nucleic acid) 就接觸到 TRL7 或 TRL9，就算沒有複製或沒有活性的病毒都會啟動 TLR_s[64-66]。

TLR_s 的訊息會經由 MyD88 蛋白傳導到以下兩個途徑：

1. 經由 IRF7 的磷酸化過程，進一步活化第一型干擾素 type I IFN 而 IFN-β和 IFN-α受到 IRF3 和/或 IRF7 的調控[67]，活化下游促炎性細胞因子(proinflammatory cytokine)。第一型干擾素在癌症治療是誘導癌細胞凋亡(apotosis)，同時經由毒殺 T 細胞(cytotoxic T cell)和計畫性樹狀細胞(programming dendritic cells)促進身體免疫的調控，要活化 TLR9 下游的分支，可以藉由 CpG ODNs 或其他 ligands 分泌第一型干擾素(IFN-α/β) ，以上是在先天免疫抗病毒最重要的機轉。TLR9 的作用在於面對體內受感染的細胞或癌細胞時，TLR9 產生固有的先天免疫反應(innate immunity)和適應性免疫反應(adaptive immunity)。TLR9 最早被認為是人體的細菌 DNA 收發器。後來發現 TLR9 還可以鑑別病毒的 DNA、黴菌和死細胞。
2. 經由 NF-κB 活化下游促炎性細胞因子(proinflamatory

cytokine)[68]。

漿細胞樣樹突細胞 (plasmacytoid dendritic cells (pDC)) 因為不含 TLR2、TLR4、TLR5 和 TLR3，所以 pDC 並不反應細菌成分[69,70]，例如：peptidoglycans, LPS, flagellin。pDC 只會認識 DNA 和 RNA 病毒。人類的 pDC 只含有 TLR7、8 和 9。哺乳類的 pDC 只含有 TLR7 和 9。pDC 上的 TLR9 可以識別 HSV-1、HSV-2 和 MCMV 病毒[71,72]。TLR7 可以識別單股 RNA 病毒，例如：流感病毒。當細胞表面接觸到這些外來物，訊號被傳到細胞內酸性內質體隔室(acid endosomal compartment)的 TLR7、TLR8，TLR9，訊號在經由銜接 TLRs 的 MyD88 往下傳，之後經由內吞作用 (endocytosis) 進入 pDC，病毒可達到溶小體(lysosomes)[73,74]。其中病毒核酸與 TRL7 與 TRL9 互動，觸發 MyD88 依賴性信號傳導途徑。重要的是，該途徑也可以由非複製的病毒或不活性的病毒所觸發。也就是說：即使不存在或沒有病毒複製，也會允許 pDC 啟動第一型干擾素的反應[75]。

在之前的研究發現：當刺激病毒或 TLR7/9 配體 (ligands)，人類和小鼠的 pDC 可以產生 10~100 倍有效的第一型干擾素[76,77]。而轉錄 IFN-β和 IFN-α基因的調節者主要是 IRF-3 和 IRF-7[78]。因此在 IRF7 有缺失的小鼠幾乎完全不能產生第一型干擾素[79]。IFN-β和 IFN-α的

調節可以經由自體分泌回饋路徑 (autocrine feedback loop)[80,81]，而 IRF7 經由回饋而產生更多的第一型干擾素。相較之下，在一般 (classical) 的樹突細胞經由 TLR7 和 TLR9 觸發分泌的是促炎性細胞因子 (pro-inflammatory cytokine) 和趨化因子 (chemokines)，而不是第一型干擾素。所以 IRF-7 在細胞樣樹突細胞 (pDC) 和在一般樹突細胞所表達產生促炎性細胞因子的能力就不一樣。如果缺少 MyD88，感染 HSV-1 或流感病毒之後，促炎性細胞因子只有部分減少。也就是說：樹狀細胞、吞噬細胞或其他型式的細胞會取代 TLR7 和 TLR9 的功用而製造第一型干擾素[82,83]。

細胞因子介入調控先天免疫 (innate immunity) 和獲得性免疫 (adaptive immunity) 的研究中，第一型干擾素是最常被討論的抗病毒物質[84,85]。然而，它們也有效的調節先天或獲得性免疫機轉。經由第一型干擾素的分泌，漿細胞樣樹突細胞 (pDC)會增強 NK 細胞和 CD8+T 細胞的細胞毒殺活性 (cytotoxicity)，並保護樹突細胞免於細胞病變 (cytopathic effect)，並協助樹突細胞抗原呈現功能 (antigen presenting function) [86]。pDC 還會分泌額外的細胞因子，例如：IL-12 和-6，它們可以和第一型干擾素合作，進一步調節免疫反應。經由分泌 IL-12 和第一型干擾素。pDC 誘導 IFN-α 分泌 NK 細胞，CD8+T 細胞和 CD4+T

helper 細胞促使清理細胞內的病原菌[87-89]。經由 IL-6 和第一型干擾素分泌，pDC 促進記憶性 B 細胞 (memory B cells) 分化成會分泌抗病毒抗體的漿細胞 (plasma cells) [90,91]。

雖然流感疫苗可以預防流感病毒，但是流感病毒的突變率太高，目前尚未證實疫苗是否可以防止未來流感的大流行。而人類社會無法全面性完整接受疫苗施打，以及無法確定經由流行病學所預測的三價流感疫苗，是否就是來年的全球流行病毒株。所以抗流感藥物的治療變成相當重要的一環。目前流感疫苗的製造是由胚胎蛋白培養。為了尋找更好的培養基，有學者發現抑制干擾素的訊號會讓流感病毒的產量增加[92]。另外，抑制 IRF7 訊號也會讓流感病毒增加[93,94]。這裡提供我們一個假說：細胞受到流感病毒感染之後，啟動細胞下游轉錄因子 IRF7 進一步產生抗病毒的干擾素。

材料與方法

病毒和細胞培養

Madin-Darby canine kidney (MDCK) 細胞和人類胎兒腎臟細胞 human embryonal kidney (293T) cells 來自生物資源保存和研究中心 (新竹，台灣) 並培養於 Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, 加州, 美國)，其中含 10% 的胎牛血清 (Invitrogen)，100 單位/cc 的抗生素 penicillin，和 250 μg/ml streptomycin B (Roche Diagnostics 公司)，之後培養在 95%空氣、5%二氧化碳的培養箱中。感染的流感病毒 A/WSN/33(H1N1)是從 American Type Culture Collection (Manassas, VA, USA) 獲得，流感病毒量是放在 MDCK 細胞上決定。MDCK 細胞放大流感病毒量，每天觀察 MDCK 細胞至 2 天後，8~9 成的 MDCK 細胞出現細胞病變 (Cytopathic effect) 後，收集上清液並離心，將此病毒液分裝於冷凍保存管內，貯於-80℃ 中備用，並以病毒定量法（Tissue culture infective dose，簡稱 TCID50）測定效價。

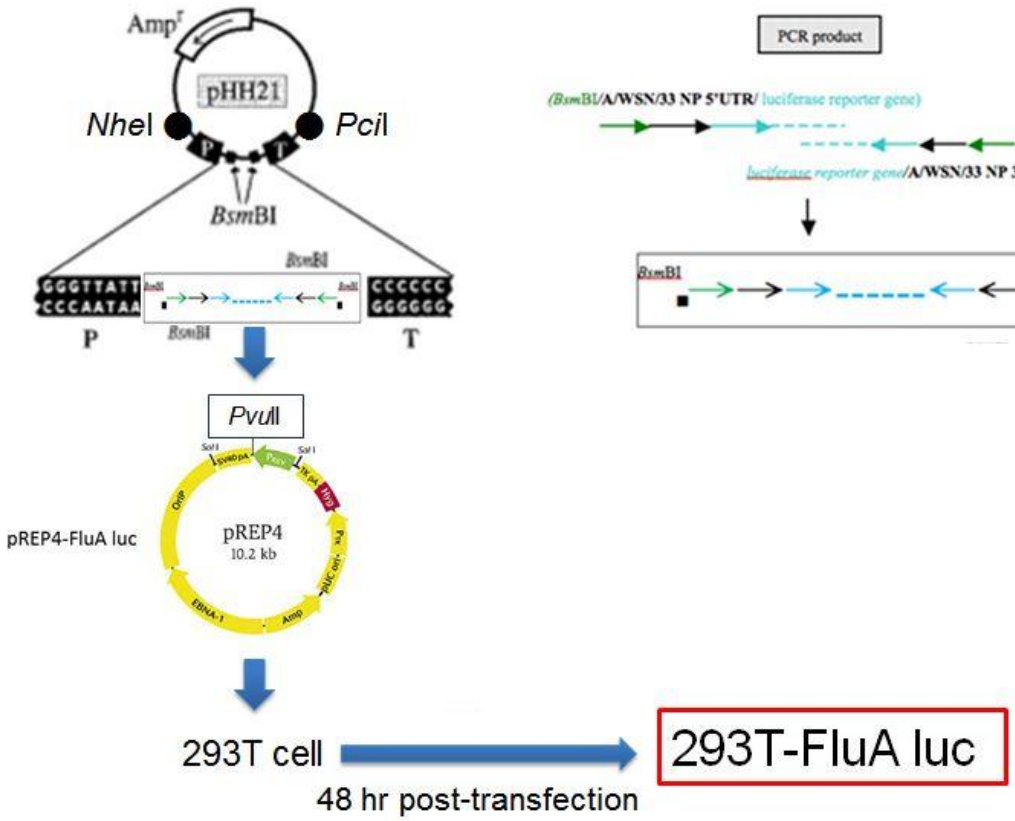
質體（plasmid）之建構

A型流感病毒冷光報告細胞的建構是使用下列的primers先行建構含有冷光酶（luciferase）之報告基因：

Lucfor:5’-ATA**CGTCTC**GGGG**AGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGT***AAAATGGAAGACGCCAAAAACATAAAG*-3’

Lucrev-5’ATA**CGTCTC**ATATT**AGTAGAAACAAGGGTATTTTTCT***TACAATTTGGACTTTCCGCCC*-3’

5’序列對應下劃線區為*Bsm*BI切點，粗體區為A/WSN/33的NP段未翻譯區域，斜體字部分冷光酶報告基因的編碼區序列。將此段DNA增幅成PCR產物。再將此含有*Bsm*BI切點的PCR產物，經*Bsm*BI剪切後，選殖到含有RNA聚合酶的啟動子（promoter），和終止子（terminator）的pHH21載體中間 (來自Prof. Earl Brown, University of Ottawa, Ottawa, Ontario, Canada)。聚合酶連鎖反應是使用Vent聚合酶（New England Biolabs, Beverly, MA）。接合(annealing) 和延展 (extension) 的狀況是利用Premier Designer v.4（Sci Ed Central, Durham, NC）來預測。聚合酶產物是利用 *Bsm*BI 切點並插入pHH21載體之中。



註：P: 促進子（human RNA polymerase I promoter）

T: 終結子（mouse RNA polymerase I terminator）

流感病毒誘導之冷光報告細胞

我們將含有 RNA polymerase I 的 promoter，流感病毒的核蛋白，冷光報告基因和 RNA polymerase I 的 terminator 的 DNA 片段經由 *Nhe*I 和

*Pci*I 限制內切酶由 pHH21 質體切下，此 DNA 片段經由 QIAquick gel extraction kid 純化，由 klenow enzyme 純化成鈍端。此時 pHH21 載體用 QIAquick 凝膠萃取試劑純化之（Qiagen, Valencia, CA）。

此一純化過後的鈍端DNA片段，克隆(cloned)入含有*Pvu*Ⅱ切點和抗生素篩選點的pREP4質體 (Invitrogen, Carlsbad, CA)，如此創造了含有表達流感病毒的pREP4冷光質體 (pREP4-FluA luc)。我們將pREP4-FluA luc轉染至293T細胞（Invitrogen, Carlsbad, CA），創造出含有流感病毒啟動子（promoter）和終止子（terminator）的冷光報告細胞：293T-FluA Luc。不同濃度的中藥或化合物加入含有流感冷光基因質體的293T細胞之後15分鐘，再加入10^5 plaque-forming unites (PFU) 的A型流感病毒，經過一小時的混合之後，利用phosphate-buffered salinec (PBS) 沖洗，再放入37℃DMEM溶液過夜。

病毒感染

A549 細胞接種到 96 孔盤($1x10^4$ 細胞/孔盤)，並在 37°C 和 5% CO_2 培養箱內 24 小時，然後將細胞用磷酸塩緩衝塩水(PBS)洗滌兩次，A 型流感病毒是用含有 0.2%牛血清蛋白的 PBS 稀釋到 10 MOI (multiplicity of infection) /100μL。細胞被感染 10 MOI 的 H1N1 A 型流感病毒之後，

用 2000 rpm 在室溫下離心一小時，細胞用 PBS 洗了兩次並加入測試抗流感藥物。之後 96 孔盤放在 33°C 和 5% CO2 培養箱內 24 小時，含有流感病毒的上清液加入 pREP4-FluA-Luc 的 293T 細胞，並在 37°C 和 5% CO2 培養箱內 24 小時。冷光素酶活性通過冷光測定系統 (Promega, 麥迪遜，威斯康辛州，美國)。

中藥對A549載體細胞的安全性測定（Growth inhibition 50%，簡稱 GI50）

稀釋過濾300種治療呼吸道感染之中藥，於96孔培養盤中加入A549細胞($2x10^3$ cell/well)，置於37℃培養箱培養24小時後，加入100µl不同濃度之中藥（1000、500、250、125µg/ml），24小時後，用1xPBS 清洗，利用wst-1（10ul wst1+90ul culture medium/ per well (450 nm)），偵側中藥對A549載體細胞之毒性。控制OD450值介於1.0~2.0之間即可完成測定。

50%細胞感染劑量（50% tissue culture infectious dose，簡稱TCID50）

第一天將MDCK細胞種到 96孔深孔盤($6x10^3$ cells / well) (1 well / 100ul DMEM)。隔天種病毒，病毒原液要先稀釋到10^{-3}~10^{-12}倍。將稀釋好的病毒液加入細胞中，1個深孔盤加入20ul 稀釋病毒液，放37℃ incubator 培養2~3天。最後利用電腦公式輸入顯微鏡觀察到的細胞病變(CPE) 之深孔盤數計算出TCID50。

篩選抑制病毒的中藥

（一）將96孔培養盤加入A549細胞(10^4 cell/well)，置於37℃培養箱培養24小時。

（二）先將舊的培養基吸取丟棄，以無菌PBS洗滌細胞兩次，以10倍稀釋(1xPBS+0.2% BSA)的流感病毒量進行病毒感染，控制組則加入100 µl DMEM含0.2%BSA，2000 rpm室溫離心1小時。

（三）移除病毒液，以無菌PBS洗滌細胞兩次，中藥萃取液以100倍及500倍稀釋後於96孔培養盤，每孔加入100 µl，每個稀釋倍數做3重複，控制組則加入100 µl DMEM含0.2%BSA，置於33℃培養箱培養24小

時。

（四）另外用96孔培養盤(poly-L lysine coating 37℃ 1hr或4℃ O/N，用1xPBS清洗2次後使用)，每孔加入293T-FluA細胞($2.5x10^4$ cell/well)，置於37℃培養箱培養24小時。

（五）24小時後，將293T-FluA細胞移除舊的培養液，以無菌PBS洗滌細胞兩次，再將 24小時培養後的A549細胞上清液轉移至293T-FluA細胞上，離心1小時2000 rpm，置於33℃培養箱培養24小時。

（六）將293T-FluA細胞取出移除上清液，加入20μl的1x cell lysis buffer，室溫作用15分鐘，加入70μl LARII，利用promega dual luciferase assay系統，計算冷光值觀察病毒抑制效果。中藥或其中之化合物萃取蛋白做法一樣，除了萃取蛋白需新鮮配製過0.45μm filter之外，中藥可事先過濾好-20度備用。

病毒斑分析（Plaque assay study）

MDCK 細胞接種到 6 孔盤($1x10^6$ 細胞/孔)，孵育在 37℃和 5%二氧化碳培養箱過夜。隔天吸除媒介質（medium），細胞用 PBS 洗滌兩次，並感染 H1N1 流感病毒 (100 PFU/孔)，病毒液以 1xPBS+0.2%BSA 進行 10 倍序列稀釋（100 μl virus + 900 μl medium）（10-3~10-7）。將已稀釋

好的病毒液取 200μl 加至六孔盤中，輕輕搖晃使病毒液均勻散布孔中並保持在冰上一小時。將 0.3% 瓊脂糖（agarose）先滅菌備用，使用前微波至完全溶解，置於 42℃水浴中使之不凝固。將 2X DMEM 及 50μg/ml 胰蛋白酶（trypsin）依比例混和後置 42℃水浴中預溫，再與 0.3%瓊脂糖混和，為避免凝固，需馬上混合用微量吸管分注。將細胞與虎杖和其活性化合物一同加入到含 0.3%瓊脂糖的 DMEM。此六孔盤放在 37℃與 5%二氧化碳培養箱 72 小時。之後，除去培養基液後，將細胞用 4%多聚甲醛固定於室溫下 1 小時。隨後用 0.1%的結晶紫染色 20 分鐘，用清水沖淨之後觀察 plaque，即細胞病變(Cytopathic effect) 的產生。

IFN-β的定量

為了 IFN-β中和性測驗，抗 IFN-β抗體 (eBioscience, San Diego, CA, USA)同時加入 10μg/ml 的流感病毒。細胞培養的上清液經 24 小時處理後，直接測量 IFN-β 的濃度。IFN-β 濃度的偵測是使用 IFN-β ELISA 試劑盒(PBL Assay Science, Piscataway, NJ, USA)。

西方墨點法

A549 細胞感染 H1N1 流感病毒並加入虎杖及其活性化合物之後，

利用西方墨點法 (Western blotting) 偵測。細胞用 PBS 清洗 3 次，並且用 mammalian protein extraction reagent 溶解 (Thermo Fisher Scientific, Inc; Rockford, IL, USA)，內含磷酸酶抑制劑和蛋白酶抑制劑。蛋白質用 12.5%的 SDS-PAGE 凝膠轉印跡到聚偏二氟乙烯膜。用來偵測 H1N1 流感病毒的血球凝集素 (hemagglutinin) 和神經胺酸酶 (neuraminidase) 抗體是使用 Balb/C3 小鼠，用 H1N1 流感病毒感染之後產生免疫之蛋白 (Fitzgerald Industries, Acton, MA, USA)，為了增加鑑別率，膜上會綴合 horseradish 過氧化物酶的抗小鼠 IgG 第二抗體。化學冷光產物是根據製造商的說明檢測 (Thermo Fisher Scientific)。每一個實驗皆個別重複 3 次。

TLR9 的抑制

為了測定 TLR9 之抑制效果，A549 細胞預先加入 TLR9 抑制劑: Super-iODN (Enzo Life Sciences, Farmingdale, NY, USA) 2 μM，三小時之後再加入流感病毒。

定量聚合酶鏈反應 (qPCR)

使用 RNA 分離試劑盒 (Qiagen, Valencia, CA, USA) 粹取所有的

RNA，5 μg RNA 經由逆轉錄而來的 RNA，將再轉變成 cDNA (Invitrogen)，qPCR 使用的 primers 和 probes 皆由商業公司設計製造(Roche, Burgess Hill, UK)。每一個 RNA 檢體使用 glyceraldehyde 3-phosphate dehydrogenase RNA 標準化。

結果

建立流感病毒冷光報告細胞

利用 UTR 趨動的報告基因的冷光反應來偵測流感病毒 polymerase 的活性，我們建構一人工的 RNA 片段，包含流感病毒基因的冷光片段，兩端由流感病毒核蛋白(NP)的 UTRs 片段控制。這段人工 RNA 片段再克隆(cloned)入含有 RNA polymerase I 的 promoter/terminator 的 pHH_2 載體，如此可以創造 RNA 的轉錄產品而不包含流感病毒額外的胺基酸片段。偵測的原理來自質體 DNA 中重建的流感病毒 polymerase 的啟動，293T 細胞感染了含有流感病毒冷光基因的片段，並且可以表達 PA, PB, PB2 和 NP。此一流感冷光報告基因系統也可以用來測量流感病毒藥物的敏感性。當含有流感冷光報告基因的 293T 細胞感染了流感病毒，並且測試潛在的抗病毒中藥和其中之化合物，同時檢測傳統使用細胞 50% 生長抑制所需的藥物濃度(GI50)測試抗病毒效力的方式。

利用冷光報告細胞篩選與呼吸道感染有關之中藥

本研究自中國醫藥大學多樣性化合物儲存庫中，篩選 300 種臨床上

治療呼吸道疾病之中藥化合物，冀望在此化合物中發現是否對流感病毒有治療效果。以往研究治療流感病毒感染的藥物是使用病毒斑試驗(Plague assay) 方式，一種新型態的抗流感化合物的研究則可能需要 5~7 天的時間，實驗過程繁瑣複雜，且容易失敗。本研究使用 293T-FluA Luc 冷光報告細胞測試 A549 細胞受 10 MOI 的 H1N1 感染流感病毒 24 小時的上清液後，利用冷光的強弱，反應中藥抗病毒的療效。並同時使用病毒斑試驗來驗證中藥治療流感病毒之後，冷光強弱的正確性。

我們發現四種中藥（虎杖、白前、薑黃、生地黃）對流感病毒有最佳抑制效果。而其中又是以「虎杖」在本實驗中有最穩定的冷光表達和最佳的抑制效果。

虎杖暨其中之化合物對載體 A549 細胞有最低抑制生長的效果

本研究利用 GI50 的原理，確定中藥是直接對流感病毒產生作用，而非因為中藥對載體細胞(A549)的毒性作用造成冷光降低，且最好的研究時間點為 A549 載體細胞感染病毒後 24 小時。A549 載體細胞感染病毒超過 24 小時之後，開始會對載體細胞產生毒性。我們發現，虎杖(Polygonum cuspitadum)對 A549 載體細胞有最低毒性（圖一）。而不同濃度的虎杖（500、250、125、62.5μg/ml）對 A549 載體細胞均有可靠的

安全性(圖二)。虎杖其中之四種化合物（A. Rubiadin、B.Resveratrol、C. Emodin、D. Polydatin）同時間加入載體細胞處理 24 小時，對載體細胞的生長抑制沒有顯著變化。發現以上以上四種物質和虎杖物質對載體 A549 細胞的毒性均低。虎杖對 A549 細胞的 GI50 的值大於 500 μg/ml，而其他四種活性化合物對 A549 細胞的 GI50 的質均大於 100μM（圖三)。

A549 載體細胞最佳操作時間與化合物實驗最佳濃度

在進行虎杖與其中之四種化合物 Resveratrol、Rubiadia、Emodin、Polydatin 的實驗之前，我們先行測試不同濃度、不同時間下，四種藥物是否會對載體細胞有毒害作用，並藉此尋找實驗最佳操作時間，結果發現：四種藥物在 24 小時，50 uM 以下對 A549 載體細胞毒性最低(圖二、圖三)。

冷光細胞對感染病毒量表達呈負相關性

本實驗使用分別稀釋 100 倍和 500 倍的虎杖(Polygonum cuspitadum)加入受流感病毒感染的 A549 載體細胞，24 小時後加入 293T-FluA Luc 冷光報告細胞，冷光報告細胞的表達量和虎杖的濃度呈明顯負相關（圖

四)。偵測虎杖的抗病毒活性是使用病毒誘導的冷光報告基因系統。10 MOI 的 H1N1 流感病毒株感染 A549 細胞之後加入虎杖的水粹物，利用此一含有 H1N1 的 A549 細胞上清液，加入含有 A 型流感的冷光報告質體 (pREP4-FluALuc) 的 293T 細胞。虎杖會抑制 A549 細胞中 H1N1 流感病毒的複製，並表現出冷光活性的降低 (圖四)，而同時虎杖的 IC50 值為 312 μg/mL。

虎杖中的化合物對於流感病毒的抑制效果

從虎杖（Polygonum cuspitadum）其中之十二種化合物之中，測試對流感病毒的抑制效果，發現其中三種化合物：Resveratrol、Rubiadia、Emodin 效果良好。兩種化合物（Rubiadin、Resveratrol），加入已經被流感病毒感染的 A549 細胞；經過 24 小時之後，取其上清液再加入 293T 冷光細胞培養 24 小時。Rubiadin 在 50μg/ml 和 Resveratrol 在 25μg/ml、50μg/ml 皆有明顯的抗病毒效果（圖五、圖七）。四種化合物中又以 Resveratrol 對流感病毒的抑制效果最佳。兩種化合物（Emodin 和 Polydatin），加入已經被流感病毒感染的 A549 細胞；經過 24 小時之後，取其上清液再加入 293T 冷光細胞培養 24 小時。經過 24 小時之後，Emodin 在 50μg/ml 有明顯的抗病毒效果，而 Polydatin 無抗病毒效果（圖

六、圖七)。虎杖與其中之三種化合物(Resveratrol、Rubiadia、Emodin)會再同時利用病毒斑實驗(plague assay study)驗證不同濃度中藥的抗病毒治療效果。虎杖中四種活性成分 (rubiadin、白藜蘆醇、大黃素和虎杖苷)分別測試是否抑制 A549 細胞中 H1N1 流感病毒複製效果。四種活性成分 (rbiadin、白藜蘆醇、大黃素和虎杖苷) 的 IC50 分別為:大於 50μM、27.4μM、37.3μM 和 大於 50μM。此一結果表明白藜蘆醇 (resveratrol) 和大黃素 (emadin) 是虎杖成分之中抑制 H1N1 流感病毒複製最有潛力的活性化合物 (圖七)。

虎杖和白藜蘆醇抑制臨床流感病毒株

為了測試中藥「虎杖」和其中之化合物在治療流感病毒時,實驗室之流感病毒株和實際臨床流感病毒株在治療成效上是否有差別? 一株自 2009 年臨床檢體獲得的 H3N2 流感病毒和 10 株自 2009 年到 2011 年之間,從臨床收集的 H1N1 流感病毒也一樣接受虎杖和白藜蘆醇的試驗(圖八、圖九),也得到相同的抑制效果。虎杖和白藜蘆醇抑制臨床的 H3N2 和 H1N1 流感病毒株的強度也和抑制實驗室 H1N1 (A/WSN/33) 流感病毒株有相同的效果。

虎杖抑制流感病毒的機轉

為了尋找中藥「虎杖」治療流感病毒時，人體抗病毒基因的變化，我們利用定量 PCR 測試四組（控制組、加藥組、加病毒組、加藥與加病毒組）抗病毒基因的變化。在測試的許多抗病毒基因中，同時加入虎杖與流感病毒組，發現 TRL3、 TRL7、 TRL8、 TRL9、 RELA、 RIPK1（圖十、圖十二），和 CASP10、CD40、IL12B、IL15、IL18、IL1B、PSTPIP1（圖十一）有上升的情況。TLR9 在加入「虎杖」後，有上升的趨勢；然而，單獨加入流感病毒之後，TLR9 上升幅度不高，加入「虎杖」與流感病毒後，TLR9 才又上升。以上顯示 TLR9 有可能為「虎杖」在人體中調節流感病毒感染的機轉（圖十、圖十二）。

虎杖、白藜蘆醇和大黃素的病毒斑減少試驗

病毒斑減少試驗 (圖十三、圖十四) 是傳統用來驗證藥物虎杖的白藜蘆醇和大黃素的抗病毒效果。病毒斑塊的減少分別顯示在 1.47mg/mL 的虎杖 (孔 2)，0.294 mg/mL 的虎杖 (孔 5)，25μM 的白藜蘆醇 (孔 3)，和 25μM 的大黃素 (孔 6)。

神經胺酸酶和血清凝集素對於 A549 細胞被流感病毒感染後，不同藥物治療後的蛋白表現量

我們近一步將「虎杖」中的化合物一一挑出，利用西方點墨分析法（Qualitative Western Blot），分析虎杖和 Resveratrol、Rubiadia、Emodin、Polydatin 分別與流感病毒感染的 A549 載體細胞作用後，發現大黃素抑制了血球凝集素(hemagglutnin) 的表達 (圖十五； lane5)。虎杖和白藜蘆醇同時抑制了血球凝集素和神經胺酸酶 (neuraminidase) 的表達 (圖十五； lane3 and lane6)。用虎杖和它的活性化合物治療在 A549 細胞上複製的 H1N1 流感病毒，同時表現出抑制 H1N1 流感病毒血球凝集素(圖十六) 和神經胺酸酶 (圖十七) 的效果。這些資料顯示出虎杖在 A549 肺癌細胞可對抗 H1N1 流感病毒的複製。

利用人類抗病毒基因 PCR array（Human antiviral response PCR array）篩選抗病毒藥物可能機轉

確定虎杖與其中三種化合物皆為神經胺酸酶抑制劑之後，利用人類抗病毒基因 PCR array，尋找中藥抑制病毒的抗病毒基因。先將每一種

化合物分四組（控制組、加藥組、加病毒組、加藥與加病毒組），在 PCR array 的 80 種抗病毒基因中發現：CASP10、CD40、IL1B、IL15、 IL18、IL12B（圖十八）、 PSTPIP1、RELA、 RIPK1、 STAT1、TLR3、 TLR7（圖十九）、TLR8、TLR9、RPL13A（圖二十）在加藥與加病毒組的抗病毒基因有上升的情況；其中加入 Resveratrol 時，TLR9 有很明顯的上升。當只加入流感病毒時，TLR9 上升則不明顯。如果同時加入流感病毒和 Resveratrol，TLR9 也同時上升。證明 Resveratrol 亦是經由 TLR9 治療流感病毒。

利用定量 PCR 驗證人類抗病毒基因 PCR array

我們進一步再用定量 PCR data 證實人類抗病毒基因 PCR array 的正確性，並發現：Resveratrol 治療受流感病毒感染的 A549 細胞之中 STAT1、TLR3、RPL13A（圖二十一）、CASP10、CD40、IL1B、IL15、IL18、IL12B、PSTPIP1（圖二十二）、RELA、RIPK1、TLR7、TLR8、TLR9（圖二十三）在加藥與加病毒組有上升的情況，顯示 Resveratrol 可能會經由這些抗病毒基因抑制病毒。

化合物誘導 TLR9 的表達

根據定量 PCR 的資料，我們發現 TLR9 可能是三種不同化合物（Resveratrol、Rubiadia、Emodin）抑制流感病毒的機轉（圖二十四）。為了評估虎杖、白藜蘆醇和大黃素抑制流感病毒的可能機轉，本研究使用 real-time PCR 分析以下四組不同情況下的抗病毒相關基因：(組 1.) A549 細胞加入 PBS 當作對照組，(組 2.) 加入抗病毒藥物， (組 3.) 加入病毒， (組 4.) 同時加入病毒和藥物。本研究發現：當 A549 細胞加入虎杖 (294 μg/mL) 、大黃素 (25μM)，或白藜蘆醇 (25μM)，TLR9 mRNA 的表達量增加。相對於 A549 細胞只有加入流感病毒的情況下，TLR9 mRNA 的表達量和對照組比較下，並沒有明顯改變 (圖二十四, 表 1)。

白藜蘆醇 (Resveratrol) 誘導 TLR9 的表達

在蛋白質表現量的部分，受 H1N1 流感病毒感染的 A549 細胞加入白藜蘆醇之後，TLR9 有上升的情況。但對於僅感染流感病毒的 A549 細胞，TLR9 的蛋白質表現量和對照組並沒有明顯改變（圖二十五）。

抑制 TLR9 會降低白藜蘆醇抗 H1N1 流感病毒的效果

Super-iODN，一個針對 TLR9 的抑制劑，在本實驗被用來評估 TLR9 對於抑制流感病毒複製的重要性。當 A549 細胞感染 10 MOI 的 H1N1 流感病毒一小時之後，加入白藜蘆醇；另一對照組則在細胞感染流感病毒後，加入白藜蘆醇和 TLR9 抑制劑。結果發現：抑制 H1N1 流感病毒的效果在加入 TLR9 抑制劑之後變差（圖二十六），顯示出白藜蘆醇抑制病毒複製的機轉中，TLR9 扮演重要的角色。

白藜蘆醇和β干擾素的協同作用抑制 A 型流感病毒複製

為了研究第一型 IFN 對於 H1N1 流感病毒複製的抑制效果，我們進行了單獨使用 IFN-β而不加白藜蘆醇，或 IFN-β同時加入白藜蘆醇的組合，資料結果用報告基因來分析。A549 細胞感染了 10 MOI H1N1 流感病毒一個小時之後，分成三組，各別加入白藜蘆醇、IFN-ß、和同時加入白藜蘆醇和 IFN-ß。實驗結果發現：IFN-ß 在加入受到感染的 A549 細胞之後，僅輕微抑制流感病毒的複製（圖二十七），但是將 IFN-ß 合併使用白藜蘆醇，抑制病毒的效果會很明顯地超過單獨使用白藜蘆醇。然而不管單獨使用 IFN-ß 或 IFN-ß 加上白藜蘆醇治療受病毒感染的 A549 細胞，只要加上 IFN-ß 中和抗體，則對 H1N1 流感病毒複製的抑制效果

就會明顯降低(圖二十八)。尋找中藥經由 TLR9 抑制流感病毒的機轉，我們發現 Interferon β有明顯上升的趨勢（表一、表二)，Interferon β和抗病毒藥物（Resveratrol）治療流感病毒似乎有加乘的效果（圖二十七)。

流感病毒感染的 A549 細胞，加入白藜蘆醇酶和 TLR9 抑制劑與否的β干擾素之表達

本實驗還偵測受感染的 A549 細胞在接受白藜蘆醇的治療之後 IFN-ß 的表達量。A549 細胞感染了 10 MOL 的 H1N1 流感病毒之後，分別三組各加入白藜蘆醇、TLR9 抑制劑，和白藜蘆醇加 TRL9 抑制劑。IFN-ß 的表達量在白藜蘆醇治療組明顯高於加入 TLR9 抑制劑和白藜蘆醇加入 TLR9 抑制劑這兩組（圖二十九)。以上結果顯示在體外實驗，白藜蘆醇合併 IFN-ß 在治療受 H1N1 感染的 A549 細胞有加成抑制病毒複製的效果。

虎杖暨其化合物治療受感染的 A549 細胞後，對於經由第一型干擾素路徑的抗病毒基因之表現

MyD88 mRNA 的表達量在虎杖的治療之下有明顯增加。MyD88 的增加來自於 TLR9 活化產生的信號傳導。本研究也發現 TLR9 活化了 IFN 的調控因子，包括：IRF3、IRF5、IRF7，以及 NF-κB 家族。 IRF5 和 IRF7 是很強的轉錄因子，兩者也可誘發 IFN-α 和 IFN-β 的表達。我們發現虎杖、白藜蘆醇和大黃素可以明顯增加 IRF5 和 IRF7 mRNA 的表達 (表三)。當感染 H1N1 流感病毒的 A549 細胞有經過虎杖治療和只有感染病毒的細胞相比較，IRF7 rnRNA 的量有 7 倍的上升 ($p<0.05$)。

本研究也同時評估 IFNα 和 IFNβ 的表現 (表三)。當受 H1N1 流感病毒感染的 A549 細胞用虎杖和白藜蘆醇的治療與沒有治療但有感染病毒的細胞相比：IFNβ mRNA 的表現量有兩倍上升的情況，但我們並沒有發現 IFNα 有明顯上升的情形。此一結果顯示了虎杖、白藜蘆醇和大黃素在治療 H1N1 感染的時候，IFNβ 在此時扮演一個重要的角色。分泌型的第一型 IFN 誘導的 myxovirus-resistance protein (MX_1) 的表達，是抑制 A 型流感病毒很重要的因子。本研究也發現：用虎杖或白藜蘆醇治療受感染的細胞和單純被感染而沒有治療的細胞相比，MX1 mRNA 的量上升了 1.3 倍($p<0.05$)。此外，interferon-induced protein IFI-15K (ISG15) 和 2', 5'-oligoadenylate synthetase (OAS2) mRNAs 的表達量和 MX_1 一樣都有上升的情形 (表三)。

討論

本研究的目的是從中國傳統中藥材及其成分中的活性化合物，找到潛在治療流感病毒的新藥物。我們建立了一個 A 型流感病毒冷光報告細胞之系統，經由此系統來篩選有潛力的新型化合物，可用來抑制流感病毒的複製。另外，透過研究這些潛在抗流感的新型化合物的分子機轉，我們可以評估新的抗病毒途徑，進而開發更多有效的治療方式。

在研究中，我們用流感病毒感染了 A549 細胞，並用不同種類的中藥材治療，結果是化合物有效抑制病毒的複製，病毒的 RNA 聚合酶的存在量就會降低，而我們的濃度依賴型冷光素酶報告系統的冷光量就會跟著降低。這裡表明一個抑制病毒複製而對應濃度依賴型降低的冷光系統，重要的是，這個篩選過程可以在 24 小時內完成，取代了舊有需 2-3 天的病毒斑成形檢測。和病毒斑成形檢測結果對照下，表明我們的冷光報告系統用來評估 A 型流感病毒的滴定是有效的。值得注意的是，本研究使用的報告細胞篩選系統和其他相類似的測定法一樣，會表現出化合物對載體細胞有毒性的情況而非抑制流感細胞，造成潛在偽陽性的結果。為了避免偽陽性的情況發生，我們進行 MTT 測試，以排除這是對載體細胞有毒的化合物。

在我們小規模篩查的項目中，排除對載體細胞有毒性的化合物，發現虎杖、白藜蘆醇和大黃素展現出對 H1N1 和 H3N2 流感病毒有顯著的抑制效果，並且對宿主細胞沒有顯著毒性。虎杖、白藜蘆醇和大黃素表現出對多種 A 型流感病毒的亞型，包括實驗室操作用的 H1N1 (A/WSN/33) 流感病毒株和臨床上獲得的 H3N2 和 H1N1 流感病毒株都有很好的抑制效果。以上兩個臨床病毒株均顯示會引起嚴重的人類呼吸系統疾病，並在二十世紀造成四次全球大流行[95]。

血球凝集素的功用是負責將流感病毒黏上宿主細胞，造成初始病毒感染的功用。而神經胺酸酶的功用是負責崩解宿主細胞接受器和子代流感病毒的連結，促進釋放子代流感病毒並進而推動流感病毒感染擴散到鄰近呼吸道上皮細胞[96,97]。我們的 western blotes 實驗表明虎杖和其中的兩個活性化合物：白藜蘆醇及大黃素會降低血球凝集素及神經胺酸酶的表達，顯示它們能夠抑制病毒的複製。

第一型干擾素系統 (IFN-α/β) 是抵禦病毒感染的第一線[98,99]。這些分子直接作用於感染病毒的細胞，從而導致細胞凋亡，並產生細胞激素和調節細胞生長、造血細胞的發展[100]。虎杖、白藜蘆醇和大黃素在宿主細胞感染流感病毒之後，只有產生 β 型干擾素。可能是 β 型干擾素僅會在受感染的細胞產生特定的抗病毒免疫反應，如果使用系統性的干

擾素，則可能會產生全身不可預期的副作用。此外虎杖、白藜蘆醇和大黃素誘導 β 型干擾素可能還同時保留它們直接對抗病毒的活性。這些協同作用可能是一種簡單而有效的方式，以減少流感病毒量。這裡還表明這些化合物的抗病毒機制可能是依賴於第一型干擾素。因此，這些數據表明先天免疫反應的激活是在這些化合物直接產生抗病毒作用之後。

A 型流感病毒的非結構蛋白 nonstructural protein 1 (NS1) 抑制了 β 型干擾素的促進因子，藉由抑制轉錄因子 IRF3 的活化而來[101]。所以，當病毒的非結構蛋白 (NS1) 存在之下，IRF3 的轉錄和 NF-κB 的激活都會失常，進一步也抑制了促炎性細胞因子 proinflammatory cytokine 和干擾素的產生[102]。實驗結果發現：IRF3 和 NF-κB 並沒有被誘導。表示虎杖、白藜蘆醇和大黃素的抗病毒機轉並不包括 NS_1 蛋白。

TRL9 有參與細胞抗病毒機轉。TRL9 激活兩個不同的途徑：一是依賴 NF-κB 的促炎性細胞因子途徑，另一是依賴 IRF7 的第一型干擾素途徑。IRF7 直接與 MyD88 信號適配，非複製的病毒基因參與和 TRL9 的互動，導致干擾素的快速分泌。在 IRF 家族中，IRF3 和 IRF7 已被確定為干擾素誘導的關鍵調節劑。然而，在之前曾有研究利用 IRF7 剔除的小鼠，無法產生干擾素的結果，證明感染 A 型流感病毒之後，IRF7 是主要負責干擾素的產生來對抗病毒[103]。我們的研究結果表明：感染病

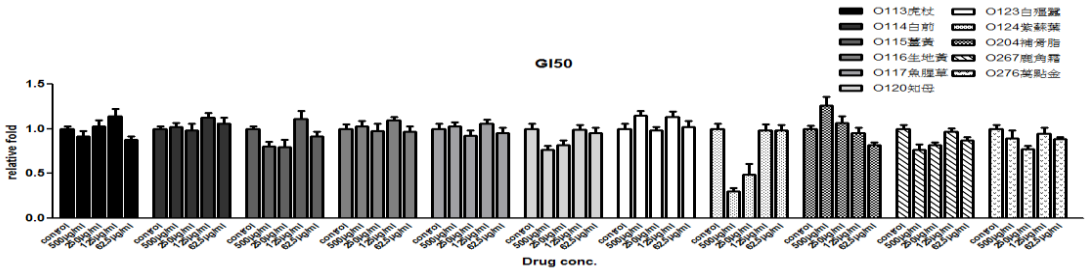
毒的宿主細胞加入虎杖、白藜蘆醇或大黃素抑制流感病毒的複製並提高了ß型干擾素的表現。此一現象可能是經由調節 IRF7 的結果。

在本實驗中，我們偵測到在藥物作用下，流感病毒的複製量下降，而 TRL9 有明顯上升的情況 ($p<0.05$)。這些數據顯示，TLR9 產量的提高和病毒誘導 IRF7 的活化，激活了 β 型干擾素的表現。總之，這些數據表明虎杖、白藜蘆醇和大黃素在 A 型流感病毒的存在下，導致 β 干擾素的產生。此一結果是經由 TRL9-MyD88-IRF7 的途徑(圖 30)。

在本實驗中，我們建立了冷光素酶報告基因系統檢測中藥材，篩選 300 種使用於呼吸道疾病的中藥材之後，我們發現三種對抗 A 型流感病毒的化合物，包括虎杖和其中兩種活性化合物：白藜蘆醇和大黃素。在流感病毒的存在下，會誘導 β 干擾素的產生。此外，這三種化合物顯示出優異的能力以誘導干擾素和 ISGs 的表達。虎杖、白藜蘆醇和大黃素抑制了 A 型流感病毒的生長，不管是實驗室的 A/WSN/33 (H1N1) 標準流感病毒株或是臨床分離出的 H3N2 和 H1N1 流感病毒株皆可以被上述三種化合物所抑制。我們發現這三種抗病毒化合物的抗病毒效果是建立在干擾素的產生及訊號的誘導。此一抗病毒的抑制效果是經由 TLR9-MyD88-IRF7 的途徑，誘導干擾素基因的表達。

最後，這項研究的結果表明：抗病毒化合物和 β 干擾素再一起使用

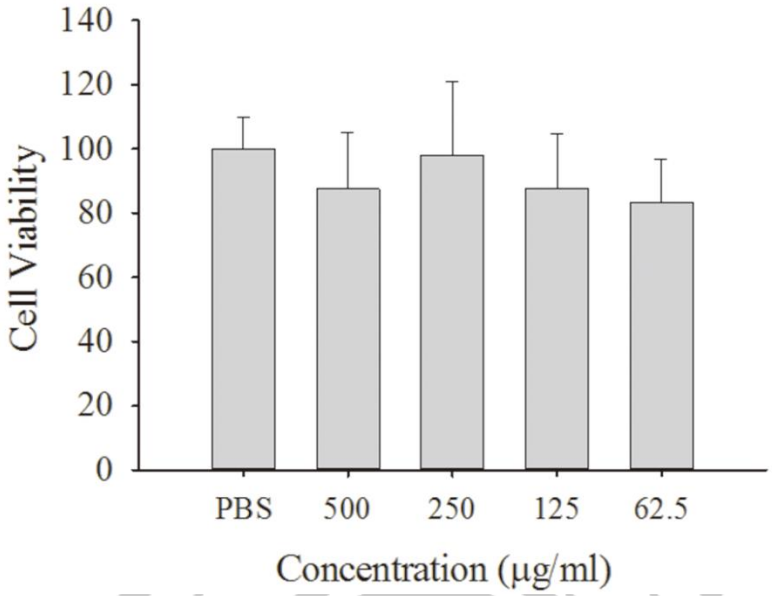
的協同作用可以對抗流感病毒的感染。這些新的見解可促使用於治療流感方法的更新，和研發抗病毒療法的進步。



圖一、中藥對載體細胞（A549 cell）的安全性測試

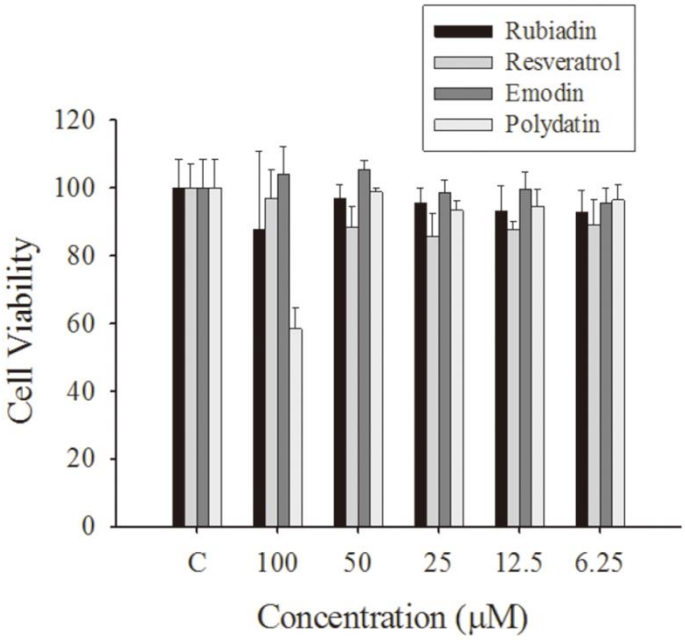
從 300 種治療呼吸道疾病的中藥裡，測試對載體細胞（A549）的毒性強弱：若中藥對載體細胞毒性越弱，表示此中藥對載體細胞的安全性越高，不會影響之後的流感病毒毒殺細胞，造成細胞減少或是中藥毒性造成細胞減少之干擾實驗的結果。每一種中藥分成四種不同濃度（62.5μg/ml, 125μg/ml, 250μg/ml, 500μg/ml），而圖示虎杖、白前、薑黃、生地黃對載體細胞有最佳安全性。

註: GI50：50%生長抑制濃度



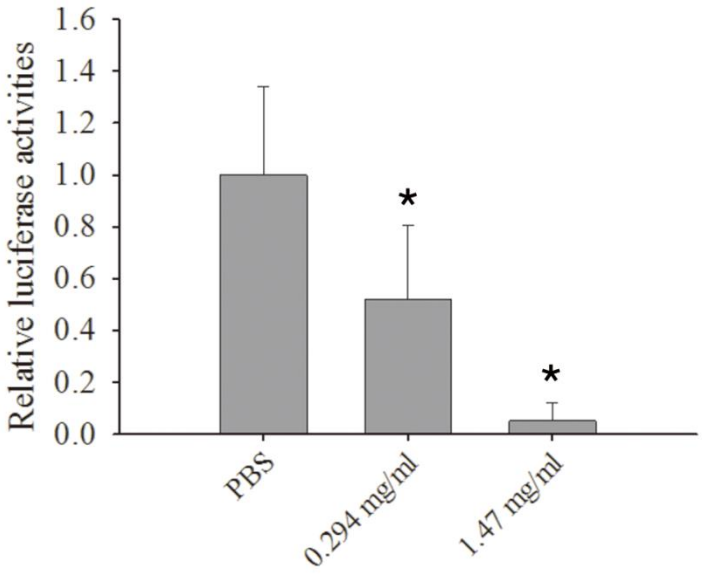
圖二、虎杖對載體細胞（A549 cell）的安全性測試

A549 細胞以 5000 細胞/孔 (cells/well) 的濃度接種到 96 孔盤中，不同濃度的虎杖加入到每個孔中 24 小時後，用 MTT 測定細胞毒性。本圖中橫軸代表不同濃度的虎杖（62.5μg/ml, 125μg/ml, 250μg/ml, 500μg/ml）；縱軸代表標準化後，虎杖對載體細胞的安全測試（GI50）。實驗數據為三次獨立實驗之平均，每次重複三次。在本實驗的處理時間內，不同濃度的虎杖在 GI50 的表現沒有顯著差異。數據以平均值±標準差表示。



圖三、四種化合物對載體細胞（A549 cell）的安全性測試

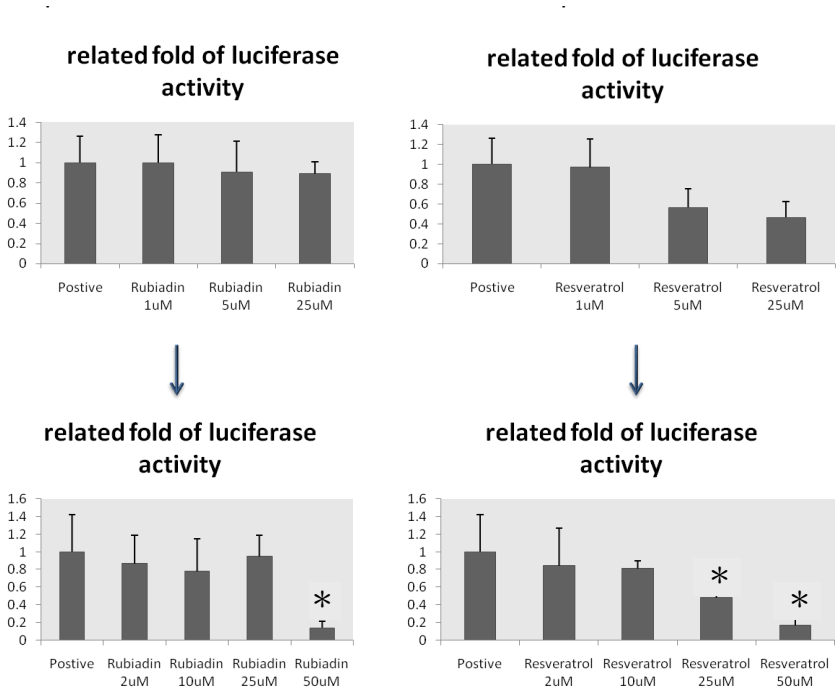
A549 細胞以 5000 細胞/孔 (cells/well) 的濃度接種到 96 孔盤中，不同濃度的 rubiadin、白藜蘆醇、大黃素和虎杖苷加入到每個孔中 24 小時後，用 MTT 測定細胞毒性。本圖中橫軸代表不同濃度的化合物；縱軸代表標準化後，四種化合物（rubiadin、 白藜蘆醇 (resveratrol)、 大黃素 (Emodin)、 虎杖苷 (Polydatin)）對載體細胞（A549 cell）生長抑制（GI50）情況。經由控制組合不同濃度（6.25μM, 12.5μM, 25μM, 50μM，100μM）的化合物，加入載體細胞處理 24 小時，對載體細胞的生長抑制沒有顯著變化。實驗數據差為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。



圖四、不同濃度的虎杖抑制流感病毒的效果

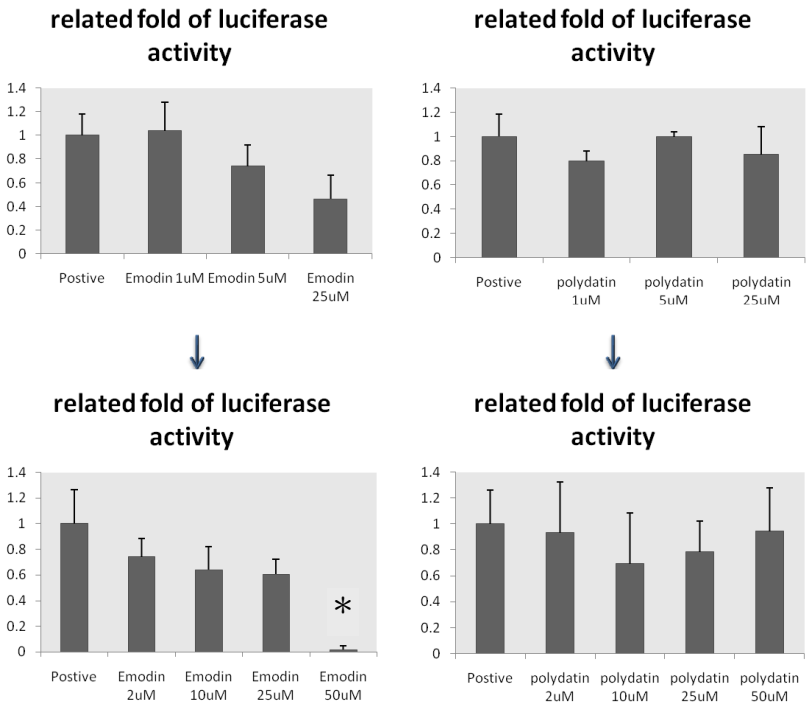
感染 10 MOI H1N1 流感病毒的 A549 細胞加入 PBS 或虎杖。利用冷光報告基因系統偵測含有複製病毒的上清液。此冷光系統是用 A 型流感病毒冷光報告基因感染 293T 細胞製造而成。本圖中橫軸代表不同濃度（0.29mg/ml 和 1.47mg/ml）的虎杖；縱軸代表表達細胞冷光活性（reporter luciferase activity）。結果顯示虎杖濃度越高，抑制流感病毒呈正比關係。

註:＊, $p < 0.05$（與控制組比較）



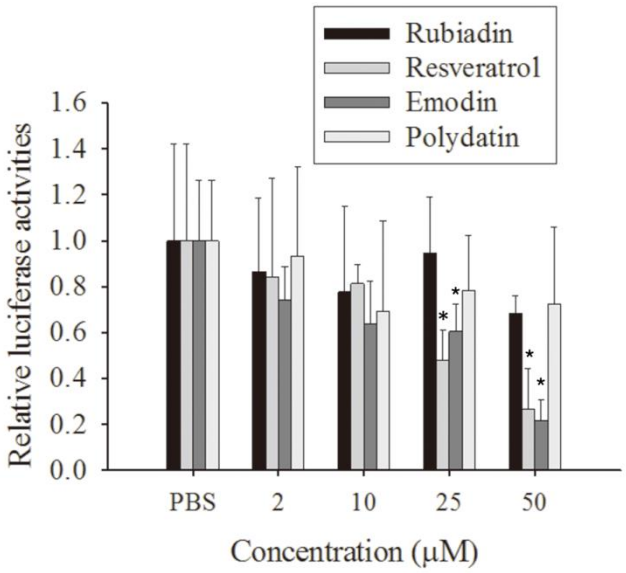
圖五、不同濃度的 Rubiadin 和 Resveratrol 對流感病毒的抑制效果

本圖中橫軸代表不同濃度的化合物；縱軸代表標準化後的冷光表達。兩種化合物（Rubiadin、Resveratrol），加入已經被流感病毒感染的 A549 細胞；經過 24 小時之後，取其上清液再加入 293T 冷光細胞培養 24 小時。Rubiadin 在 50μg/ml 和 Resveratrol 在 25μg/ml、50μg/ml 皆有明顯的殺菌效果。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊$p < 0.05$（與同時間的 control 組比較）。



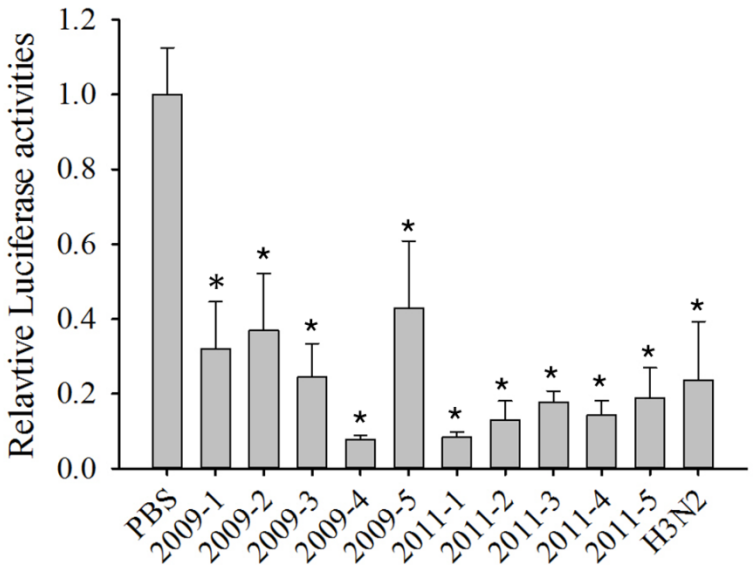
圖六、不同濃度的 Emodin 和 Polydatin 對流感病毒的抑制效果

本圖中橫軸代表不同濃度的化合物；縱軸代表標準化後的冷光表達。兩種化合物（Emodin 和 Polydatin），加入已經被流感病毒感染的 A549 細胞；經過 24 小時之後，取其上清液再加入 293T 冷光細胞培養 24 小時。經過 24 小時之後，Emodin 在 50μg/ml 有明顯的殺菌效果，而 Polydatin 無殺菌效果。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊$p < 0.05$（與同時間的 control 組比較）。



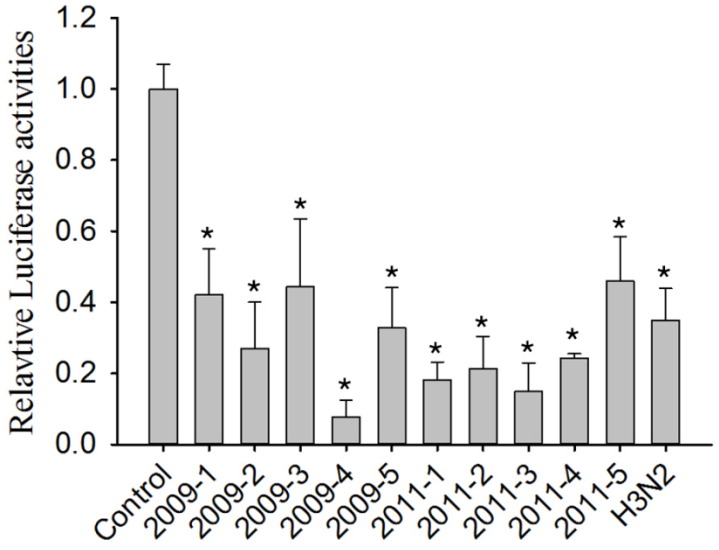
圖七、虎杖的四種不同濃度化合物對流感病毒的抑制效果

本圖中，橫軸代表不同濃度的化合物；縱軸代表標準化後的冷光表達。PBS 或四種化合物同時以不同濃度加入已經被 10 MOI H1N1 流感病毒感染的 A549 細胞；經過 24 小時之後，取其上清液再加入 293T 冷光報告細胞培養 24 小時。白藜蘆醇 (Resveratrol)、大黃素 (Emodin) 在 25μg/ml 和 50μg/ml 皆有明顯的殺菌效果。實驗數據為三次獨立實驗之平均，每次重複三次。數據是以一式三份進行的 3 個獨立實驗之平均值±標準差。星號表示為對照組和藥物處理的樣品之間配對比較所計算出的 P 值都小於 0.05。



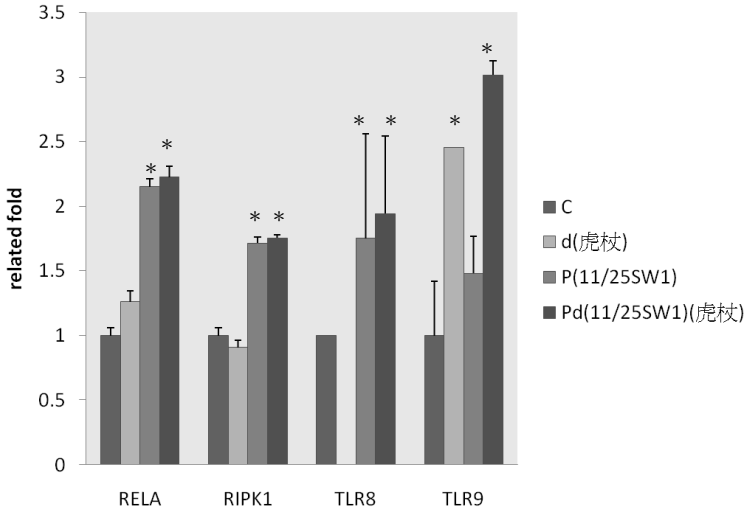
圖八、虎杖水萃物明顯抑制感染 A549 細胞的臨床流感病毒株

A549 細胞感染了 10 MOI 的 H1N1 臨床流感病毒株之後，分別加入 PBS (對照組) 和虎杖 (0.3mg/ml) 感染 24 小時之後，取其培養皿上清液加入含有 A 型流感病毒冷光報告系統的 293T 細胞，觀察冷光表現量。



圖九、白藜蘆醇明顯抑制感染 A549 細胞的臨床流感病毒株

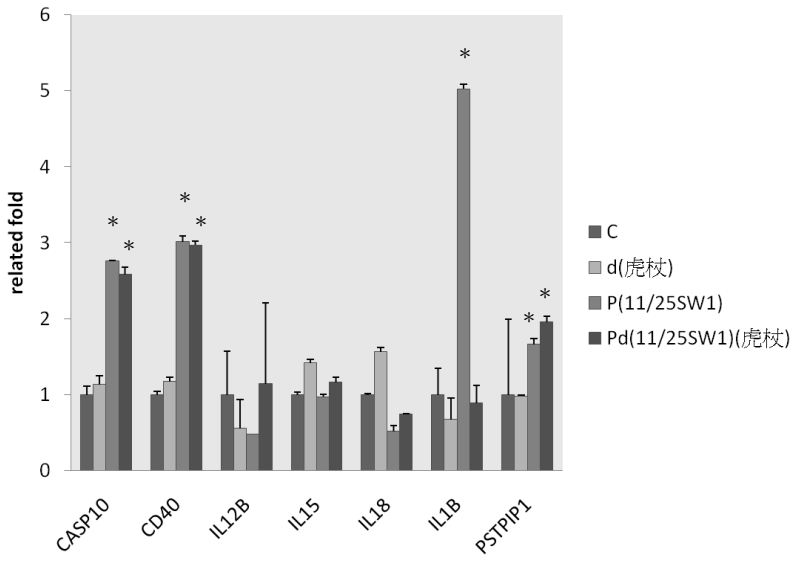
A549 細胞感染了 10 MOI 的 H1N1 臨床流感病毒株之後，分別加入 PBS (對照組) 和白藜蘆醇 (25 μM) 感染 24 小時之後，取其培養皿上清液，加入含有 A 型流感病毒冷光報到系統的 293T 細胞，觀察冷光表現量。



圖十、觀察虎杖對抗病毒基因表現之影響 (Q-PCR data)

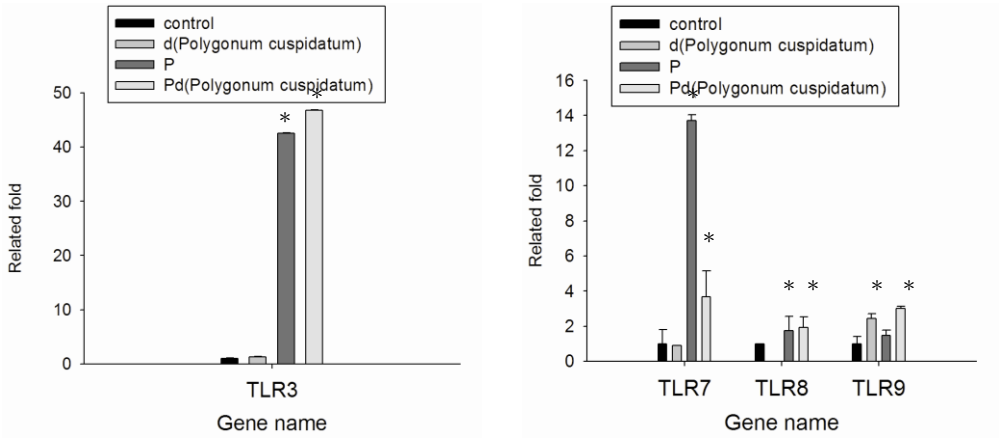
本實驗將載體細胞（A549）分別加入虎杖（d）、加入流感病毒（p）、同時加入虎杖與流感病毒（pd），作用 24 小時後偵測抗病毒基因之表現。結果顯示：除了 TLR9 在加入流感病毒組沒有明顯上升外，其他細胞激素在加入流感病毒組或同時加入虎杖和流感病毒組均上升。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。

＊p <0.05（與同時間的 control 組比較）。



圖十一、觀察虎杖對抗病毒基因表現之影響 (Q-PCR data)

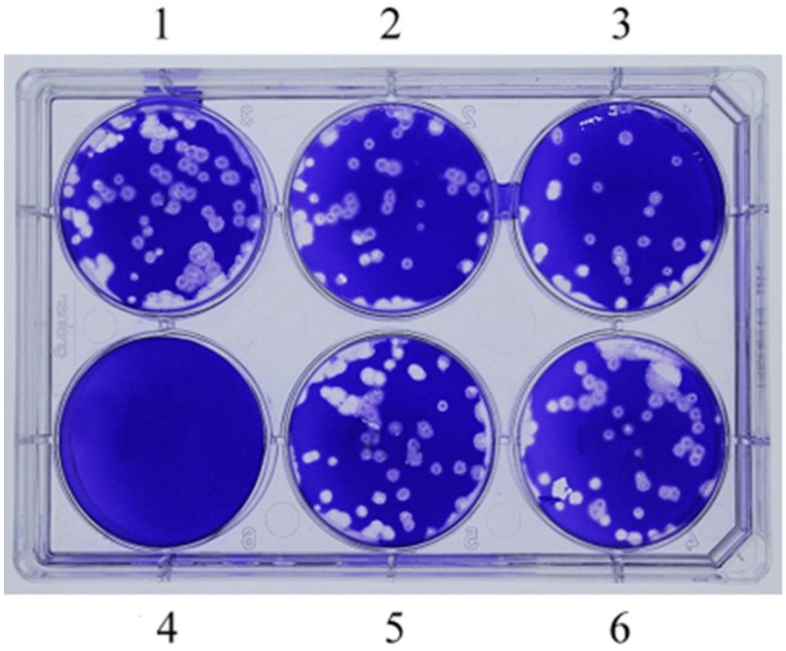
本實驗將載體細胞(A549)分別加入虎杖(d)、加入流感病毒(p)、同時加入虎杖與流感病毒(pd),作用 24 小時後偵測抗病毒基因之表現。結果顯示：CASP10，CD40，IL1B，PSTPIP1 在加入流感病毒組或同時加入虎杖和流感病毒組均上升。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊p <0.05（與同時間的 control 組比較）。



圖十二、觀察虎杖對抗病毒基因表現之影響 (Q-PCR data)

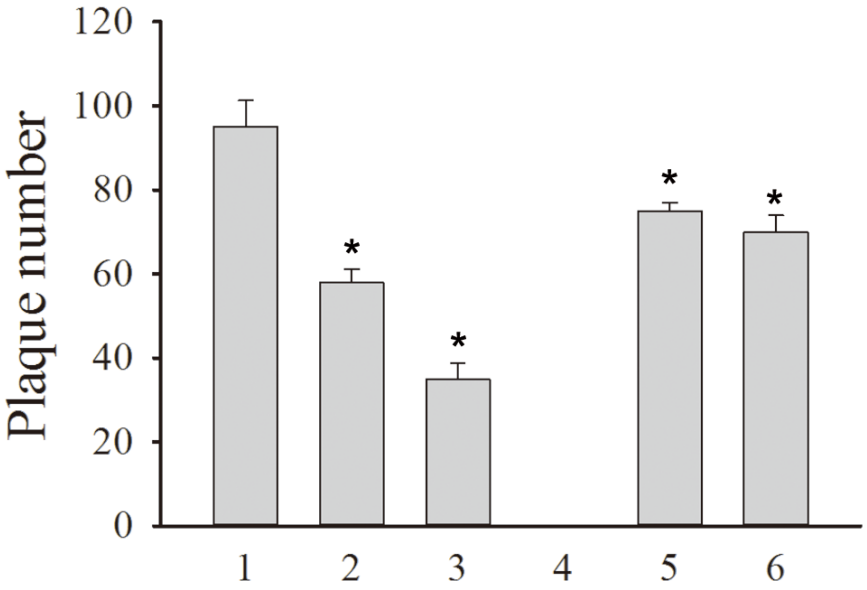
本實驗將載體細胞（A549）分別加入虎杖（d）、加入流感病毒（p）、同時加入虎杖與流感病毒（pd），作用 24 小時後偵測抗病毒基因之表現。結果顯示：TLR9 在加入流感病毒組沒有明顯上升外，TLR3，TLR7，TLR8 在加入流感病毒組或同時加入虎杖和流感病毒組均上升。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值 ± 標準差表示。

＊p <0.05（與同時間的 control 組比較）。



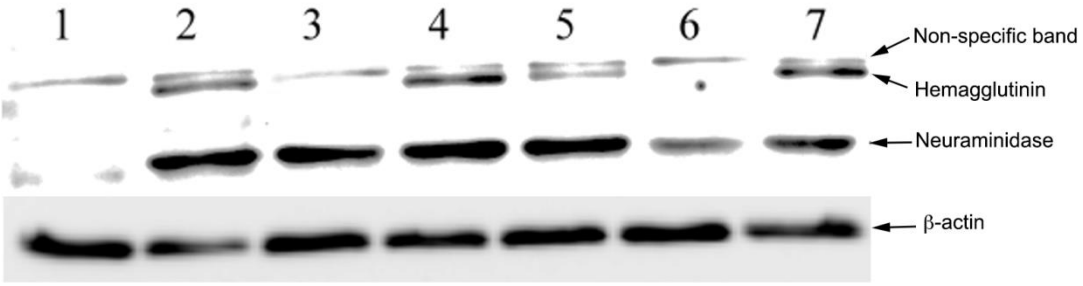
圖十三、虎杖、白藜蘆醇和大黃素的病毒斑減少試驗

MDCK 細胞接種到六孔盤 ($1x10^6$ 細胞/孔)，並感染 H1N1 流感病毒(100 PFU/孔)。此細胞分別加入虎杖及其活性化合物，並培養於 Dulbecco's 改良的 Eagle 培養基，內含 0.3% 瓊脂糖。72 小時後，將病毒斑通過用 0.1% 的結晶紫染色來確定。其中，編號孔 1：病毒；編號孔 2：病毒加虎杖 (1.47mg/ml)；編號孔 3：病毒加白藜蘆醇 (25 μM)；編號孔 4：控制組；編號孔 5：病毒加虎杖 (0.294mg/ml)；編號孔 6：病毒加大黃素 (25 μM)。



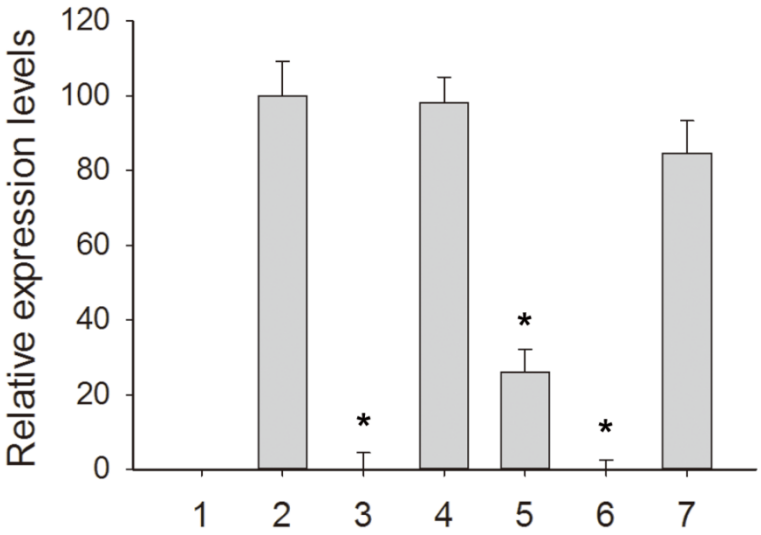
圖十四、虎杖、白藜蘆醇和大黃素病毒斑減少試驗 (接續圖十三)

結果是三次獨立實驗的平均值±標準差，星號表示所計算出的 P 值為第一組和 2、3、5、6 組比較小於 0.05 。



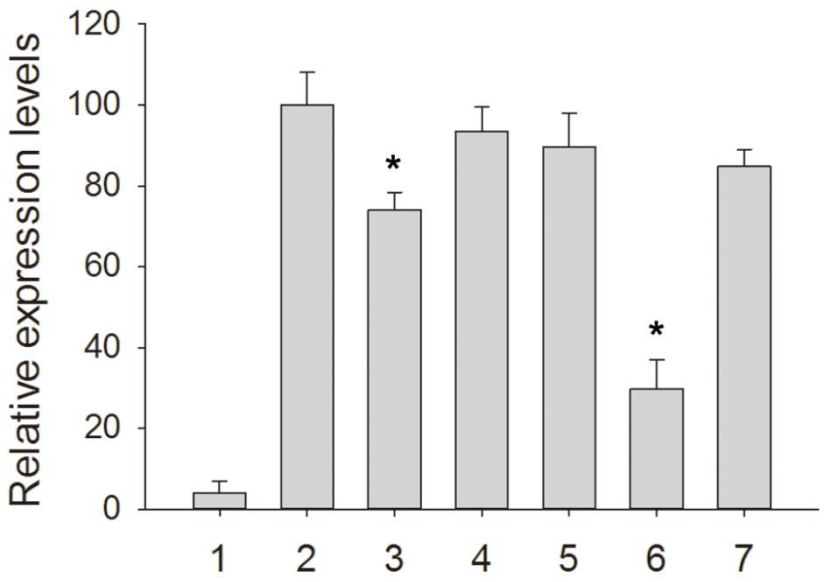
圖十五、神經胺酸酶和血清凝集素對於 A549 細胞被流感病毒感染後，不同藥物治療後的蛋白表現量之影響

H1N1 流感病毒感染了 A549 細胞之後，接受虎杖和其活性成分的治療。感染 24 小時之後，收集細胞的上清液，Lane 1 為對照組；Lane2：僅加入病毒；Lane3：加入虎杖 (1.47mg/ml)；Lane4：rubiadin (25 μM)；Lane5：大黃素 (25 μM)；Lane6：白藜蘆醇 (25 μM)；Lane7：polydatin (25 μM)；縱軸代表各組加入 A549 載體細胞後，經過 24 小時處理，神經胺酸酶（NA）和血清凝集素（HA）的蛋白質表現量。結果顯示：同時加藥（虎杖、Resveratrol、Emodin）和加病毒組的嗜血酶原和神經胺酸酶（NA）表現量有下降。β-actin 的表現量為 internal control。表示的西方點墨法為三個獨立實驗的其中之一。



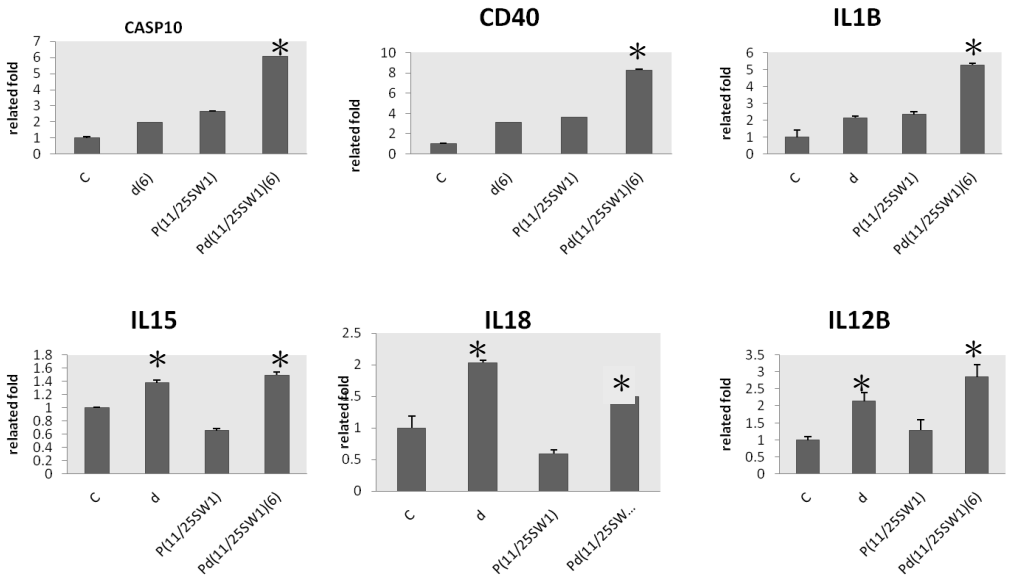
圖十六、血清凝集素對於 A549 細胞被流感病毒感染後，不同藥物治療後的蛋白表現量之影響 (接續圖十五)

由西方點墨法分析嗜血酶原的表達，將其密度定量分析。



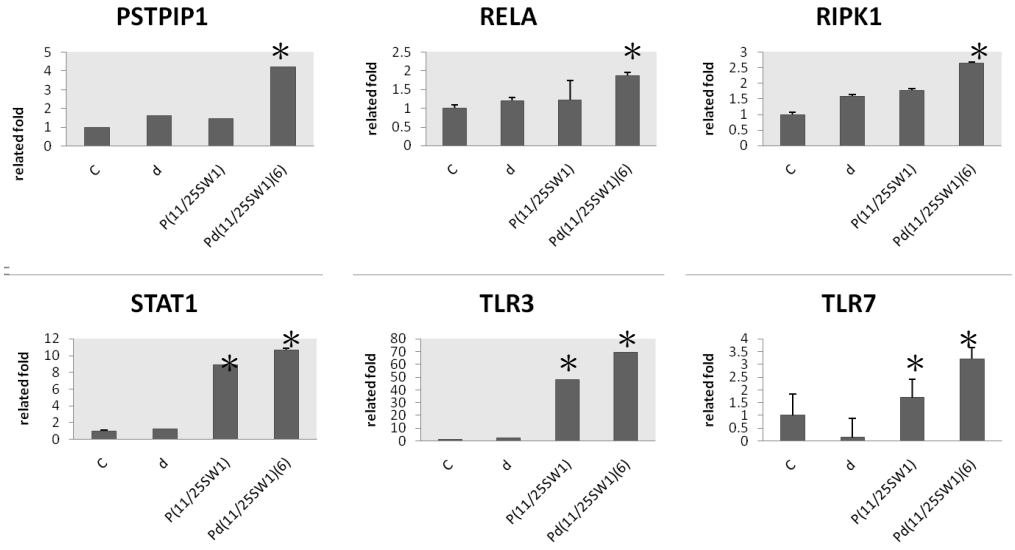
圖十七、神經胺酸酶對於 A549 細胞被流感病毒感染後，不同藥物治療後的蛋白表現量之影響（接續圖十五）

西方點墨法分析神經胺酸酶的表達，將其密度做分析。結果是：3 個獨立實驗的平均值 ± 標準差。



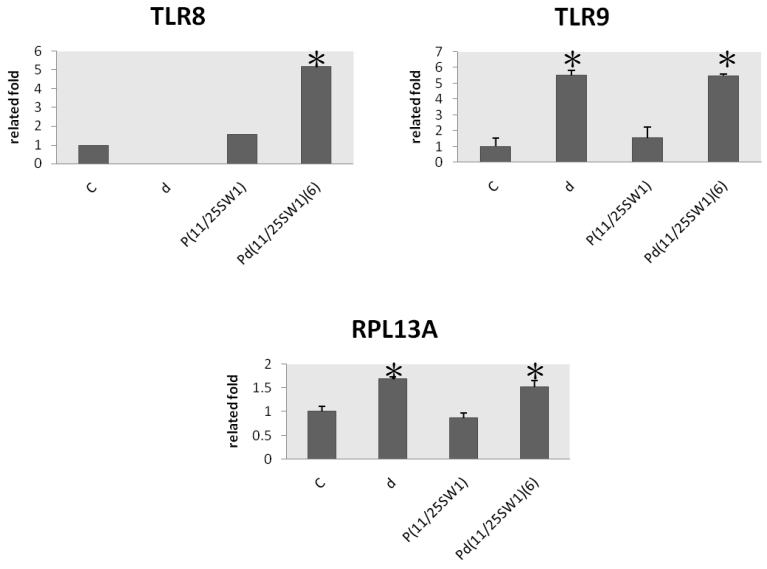
圖十八、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（PCR array data）

本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後細胞激素的生成量。結果發現：圖示的抗病毒基因在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊p <0.05（與同時間的 control 組比較）。



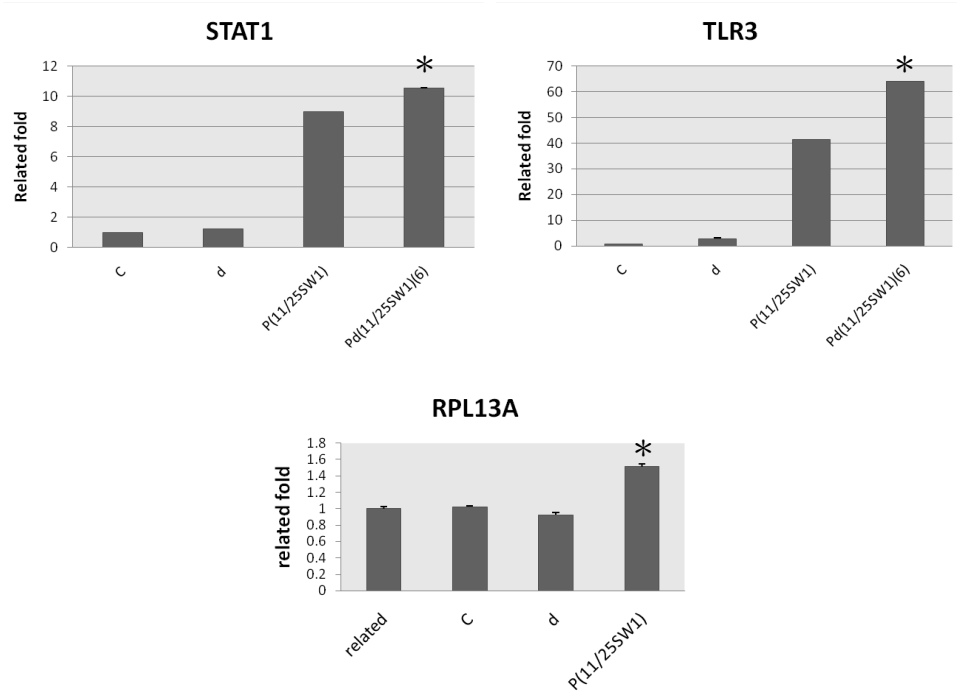
圖十九、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（PCR array data）

本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後抗病毒基因之表現。結果發現：圖示的抗病毒基因在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊p <0.05（與同時間的 control 組比較）。



圖二十、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（PCR array data）

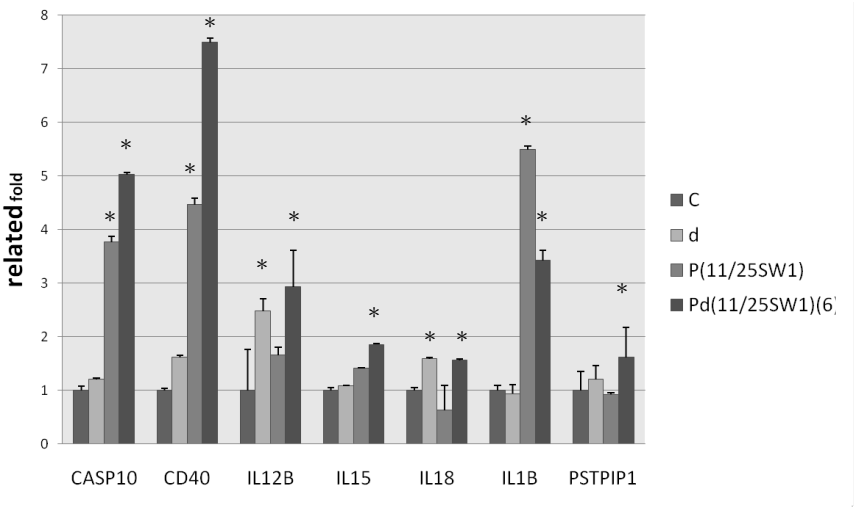
本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後抗病毒基因之表現。結果發現：圖示的抗病毒基因在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊p <0.05（與同時間的 control 組比較）。



圖二十一、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（Q-PCR data）

本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加流感病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後抗病毒基因之表現。結果發現：圖示的抗病毒基因，在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。

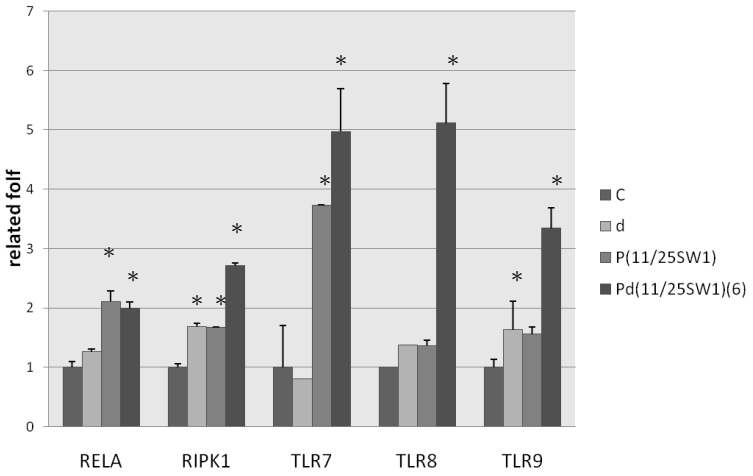
＊p <0.05（與同時間的 control 組比較）。



圖二十二、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（Q-PCR data）

本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加流感病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後抗病毒基因之表現。結果發現：圖示的抗病毒基因，在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。

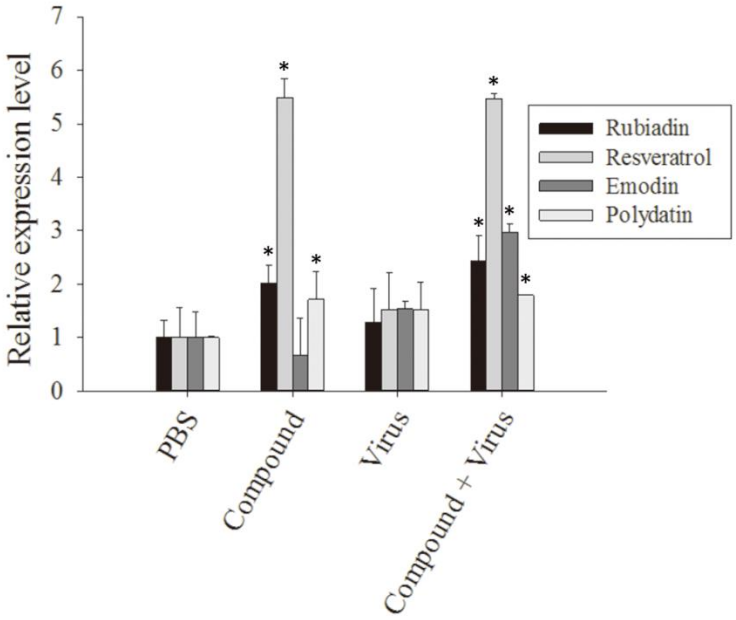
＊$p < 0.05$（與同時間的 control 組比較）。



圖二十三、流感病毒感染的 A549 細胞在接受 Resveratrol 治療後，抗病毒基因表現之影響（Q-PCR data）

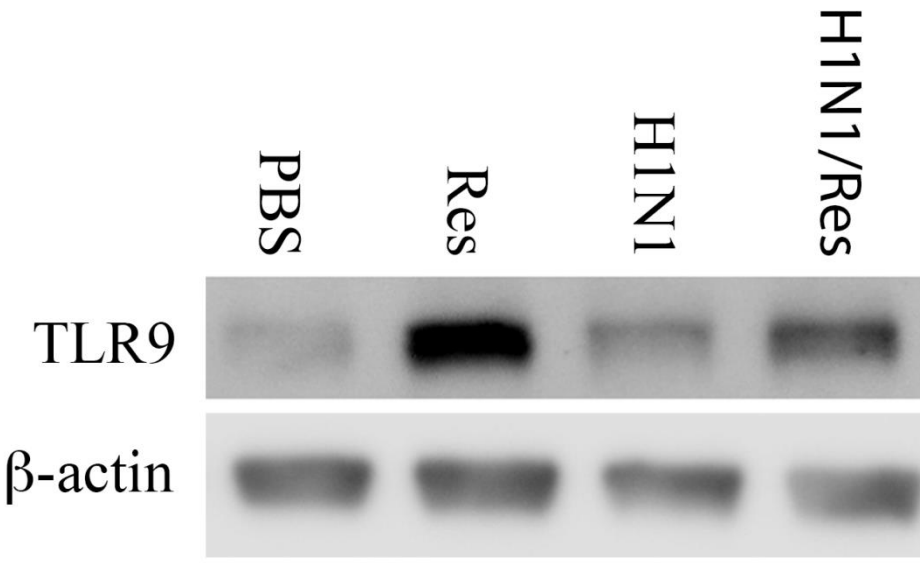
本圖中橫軸代表控制組（不加藥，不加病毒）、加藥組（Resveratrol）、加流感病毒組、同時加藥加流感病毒組，各組加入 A549 細胞後經過 24 小時處理；縱軸代表標準化後抗病毒基因之表現。結果發現：圖示的抗病毒基因，在同時加藥加病毒組有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值 ± 標準差表示。

＊p <0.05（與同時間的 control 組比較）。



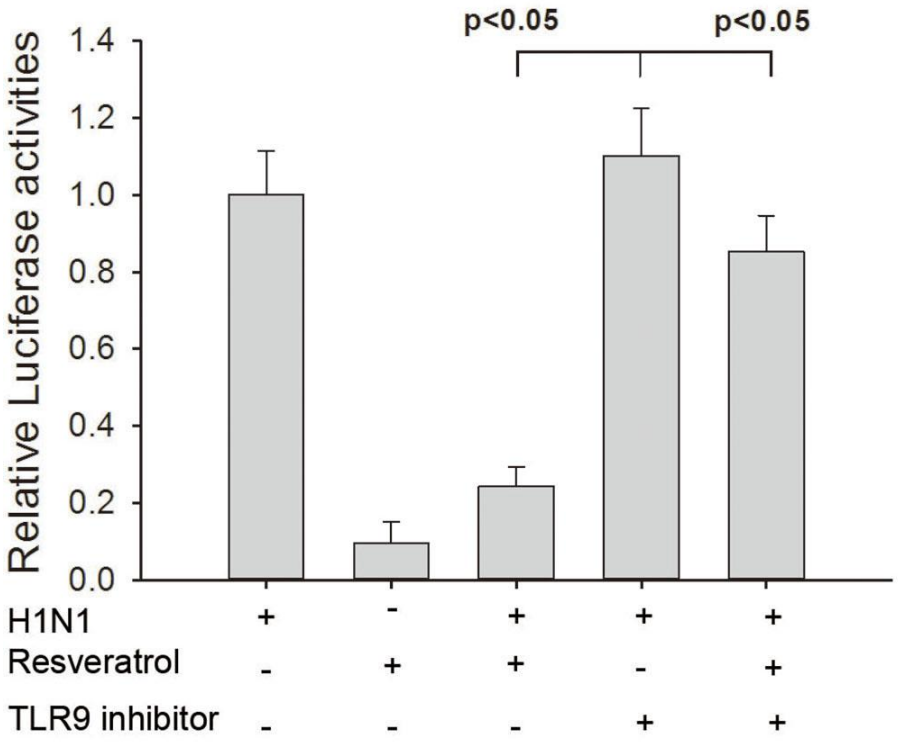
圖二十四、白藜蘆醇 (Resveratrol) 誘導 TLR9 的表達

A549 細胞加入虎杖的四種活性化合物之後，粹取出的 RNAs 利用 real-time PCR 來測量 TLR9。橫軸代表控制組（不加藥，不加病毒）、加藥組（Rubiadin、Resveratrol、Emodin、Polydatin）、加病毒組、同時加藥加流感病毒組；縱軸代表標準化後 TLR9 的生成量。四組經過 24 小時處理後，A549 細胞在加藥組（Rubiadin、Resveratrol）和同時加藥 Rubiadin、Resveratrol、Emodin，加病毒組的 TLR9 有上升的情況。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊$p < 0.05$（與同時間的 PBS 對照組比較）。



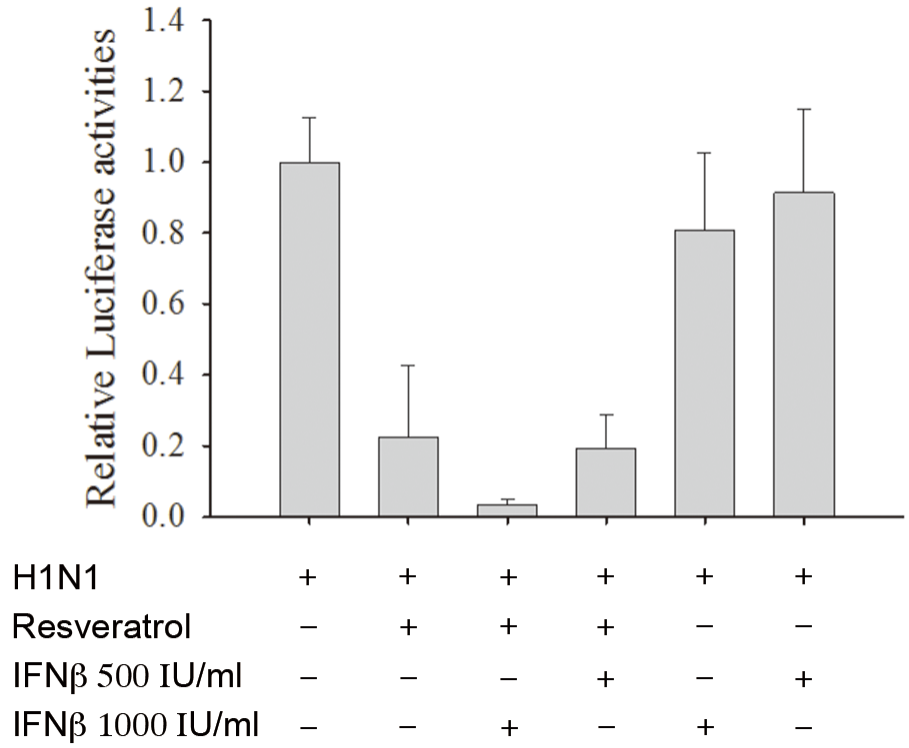
圖二十五、白藜蘆醇 (Resveratrol) 誘導 TLR9 的表達量增加

A549 細胞分別加入 PBS (對照組)，白藜蘆醇 (25 μM)、H1N 流感病毒、H1N1 流感病毒和白藜蘆醇 (25 μM)，以上四組均在感染後 24 小時萃取細胞。顯示的結果是來自三個獨立實驗的西方點墨法的其中之一。



圖二十六、抑制 TLR9 會降低白藜蘆醇抗 H1N1 流感病毒的效果。

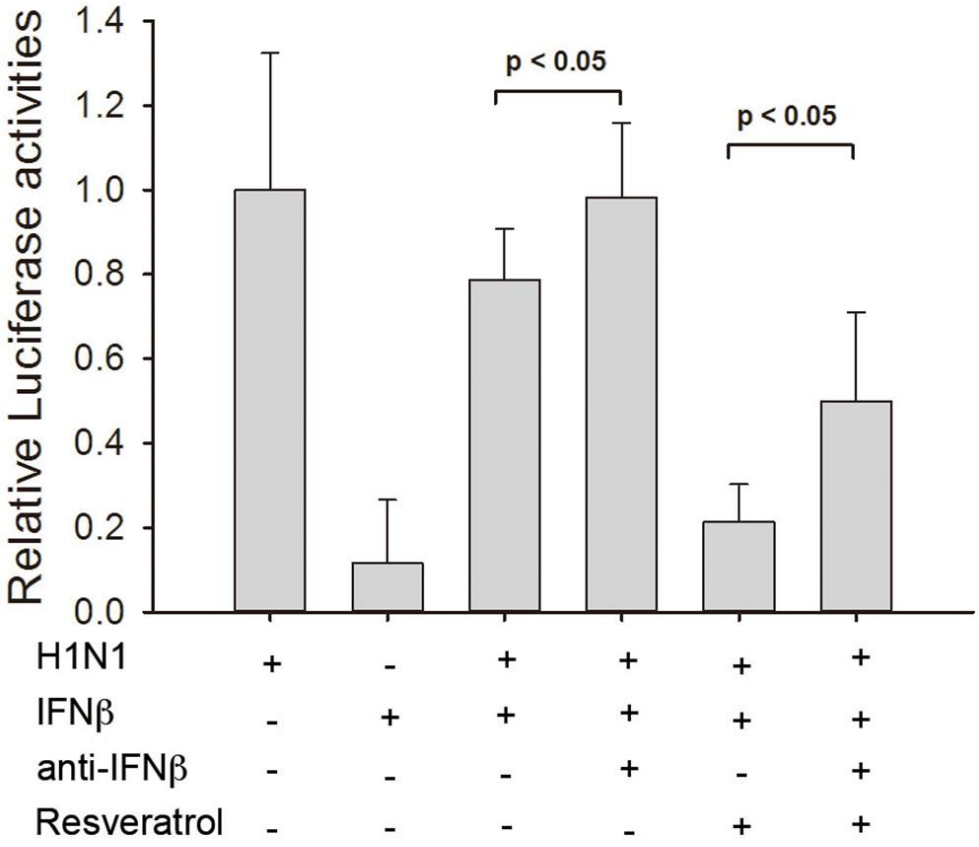
A549 細胞在感染流感病毒前 3 小時，加入 TLR9 抑制劑，在感染 24 小時之後和沒有加入 TLR9 抑制劑組作比較，分別取其培養皿的上清液測定在 TLR9 抑制劑的存在下，A 型流感病毒的複製效果。



圖二十七、白藜蘆醇和β干擾素的協同作用抑制 A 型流感病毒複製

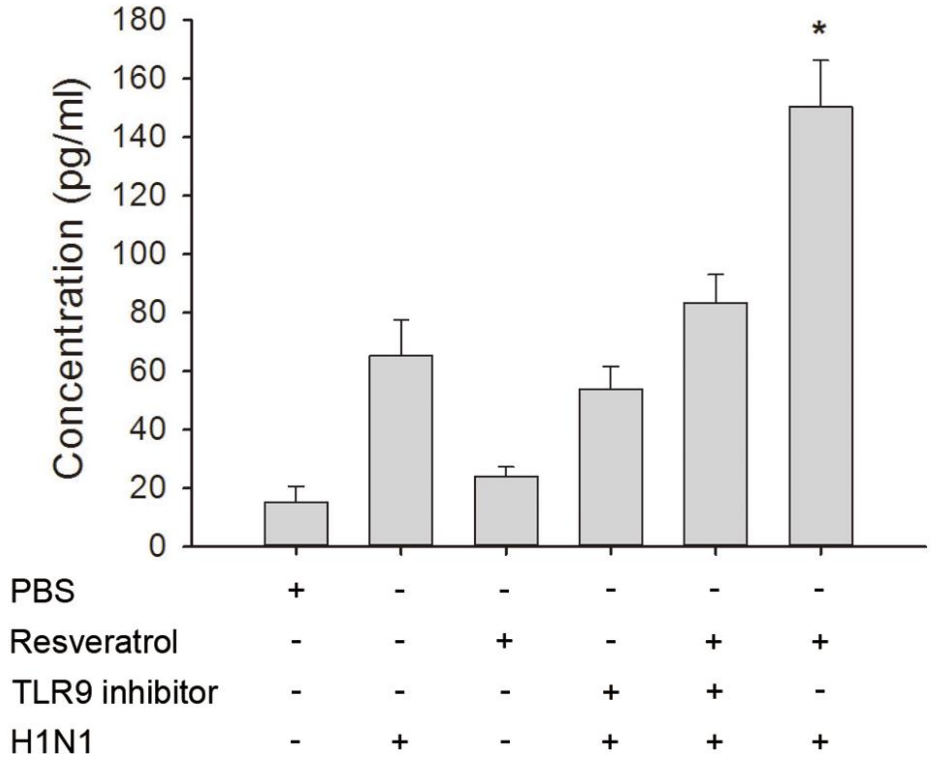
加入 A549 細胞的不同治療方式如圖示。實驗後的培養上清液中的流感病毒偵測是使用 295T 細胞感染 A 型流感冷光報告系統。利用 Student's t-tests 的 P 值配對比較白藜蘆醇 (25μM)；白藜蘆醇 (25μM) 加入β干擾素 (1000 IU/mL)；白藜蘆醇 (25μM) 加入β干擾素(500 IU/mL) 和控制組比較 P 值皆小於 0.05。縱軸代表標準化後流感病毒被抑制的程度。A549 細胞加入 Resveratrol 或β型干擾素，或同時加入

Resveratrol 和β型干擾素處理 24 小時。流感病毒被抑制的程度，在同時加入 Resveratrol 和β型干擾素的情況下，病毒抑制有加乘的效果。實驗數據為三次獨立實驗之平均，每次重複三次。數據以平均值±標準差表示。＊p<0.05（與同時間的 control 組比較），被認為有顯著意義。



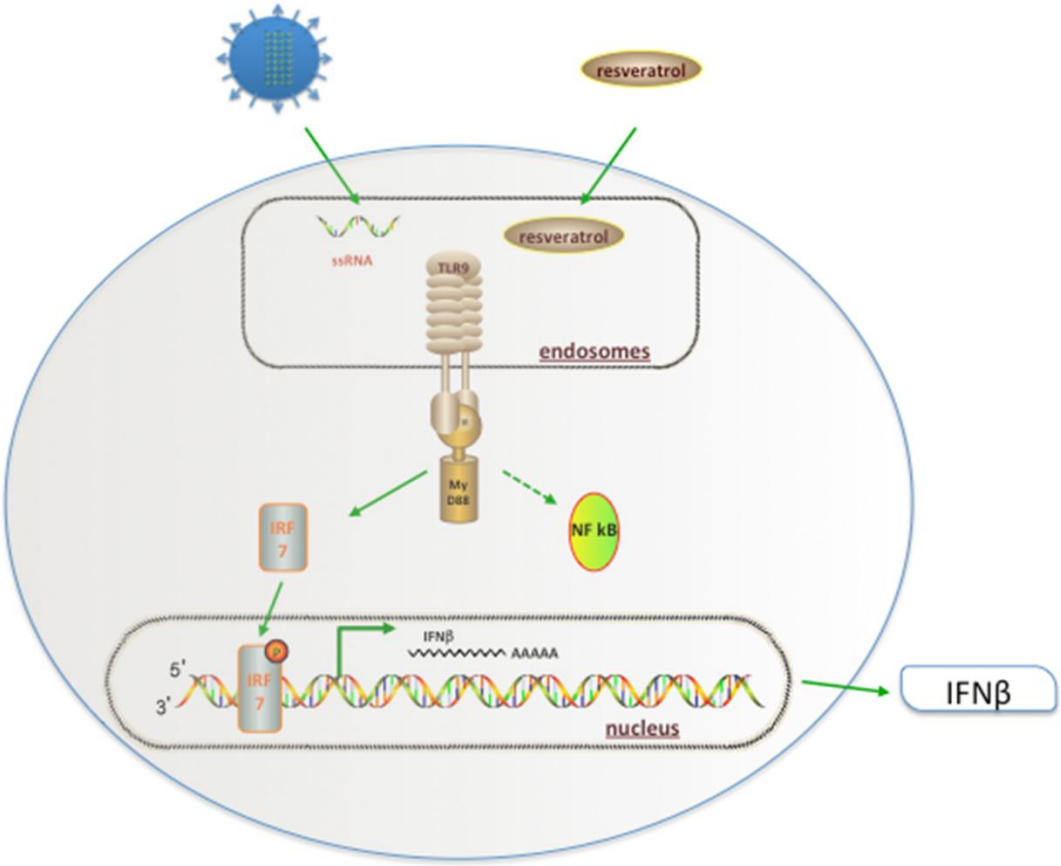
圖二十八、β干擾素抗體的中和劑減少白藜蘆醇的抗病毒活性

A549 細胞感染了 10 MOI H1N1 流感病毒之後，加入不同的試劑(如圖示)，24 小時後取其培養皿上清液的複製病毒，利用 293T 冷光偵測流感病毒報告系統，測定在β干擾素和抗體的存在下，A 型流感病毒的複製量。



圖二十九、H1N1 流感病毒感染的 A549 細胞，加入白藜蘆醇酶與否的 β 干擾素之表達

加入 A549 細胞的試劑如圖示。在感染 24 小時之後取其細胞培養上清液，利用 ELISA 測量 β 干擾素的濃度，配對比較 H1N1 和 H1N1 加入白藜蘆醇的 P 值小於 0.05，有顯著意義。



圖三十、虎杖和其活性化合物的抗病毒機轉

虎杖、白藜蘆醇、大黃素進入到受流感病毒感染的細胞之後，通過 TLR9 活性促發炎細胞因子，TLR9 的信號通過 MYD88 之後，導致兩種不同的活化途徑。虎杖、白藜蘆醇和大黃素經由第七干擾素調節因子 (IRF7) 的途徑，而不是經由 NF-κB 的通路。經由 IRF7 的磷酸化和轉錄活化促發炎細胞因子之後，激活第一型干擾素的產生。

表一、虎杖治療受感染的 A549 細胞後，對於經由第一型干擾素路徑的抗病毒基因之表現

Gene name	GeneBank	Symbol	C	d (Polygonum Cuspidatum)	P	Pd (Polygonum Cuspidatum)
Interferon, alpha 1	NM_024013	IFNA1	1	0.689	1.489	1.074
Interferon, beta 1	NM_002176	IFNB1	1	0.268	591.941	992.779
Interferon regulatory factor 3	NM_001571	IRF3	1	1.611	1.083	3.247
Interferon regulatory factor 5	NM_001098629	IRF5	1	2.527	0.806	4.015
Interferon regulatory factor 7	NM_001572	IRF7	1	3.997	88.077	632.994
ISG15 ubiquitin-like modifier	NM_005101	ISG15	1	1.105	1126.327	2340.693
Myxovirus (influenza virus) resistance 1	NM_002462	MX1	1	1.801	766.324	1026.096
Myeloid differentiation primary response gene (88)	NM_002468	MYD88	1	1.782	6.885	26.097
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_003998	NFKB1	1	0.809	2.787	1.487
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_020529	NFKBIA	1	2.418	8.724	27.944
2'-5'-oligoadenylate synthetase 2	NM_002535	OAS2	1	0.335	2585.988	2055.067
Toll-like receptor 3	NM_003265	TLR3	1	1.368	42.570	46.743
Toll-like receptor 7	NM_016562	TLR7	1	0.902	13.708	3.680
Toll-like receptor 8	NM_138636	TLR8	1	-	1.753	1.941
Toll-like receptor 9	NM_017442	TLR9	1	2.456	1.480	3.015
Tumor necrosis factor	NM_000594	TNF	1	1.161	45.829	47.762
TNF receptor-associated factor 3	NM_003300	TRAF3	1	0.818	1.408	1.200
TNF receptor-associated factor 6	NM_004620	TRAF6	1	0.885	1.249	1.001

本實驗將載體細胞（A549 cell）分別加入虎杖、加入流感病毒、同時加入虎杖和流感病毒作用 24 小時後，偵測經由第一型干擾素路徑（type 1 Interferon pathway）抗病毒基因之表現。結果 Interferon, betal 1、Interferon regulatory factor 7、ISG15 ubiquitin-like modifier、Myxovirus(influenza virus) resistance 1、2’-5’-oligoadenylate synthetase 2 在同時加入虎杖和流感病毒組有上升的情況。

表二、Emodin 治療受感染的 A549 細胞後，對於經由第一型干擾素路徑的抗病毒基因之表現

Gene name	GeneBank	Symbol	C	d (Emodin)	P	Pd (Emodin)
Interferon, alpha 1	NM_024013	IFNA1	1	0.980	2.390	1.170
Interferon, beta 1	NM_002176	IFNB1	1	0.300	656.577	492.251
Interferon regulatory factor 3	NM_001571	IRF3	1	1.751	1.240	1.334
Interferon regulatory factor 5	NM_001098629	IRF5	1	2.114	0.638	1.448
Interferon regulatory factor 7	NM_001572	IRF7	1	4.500	81.613	134.153
ISG15 ubiquitin-like modifier	NM_005101	ISG15	1	1.812	1101.436	501.811
Myxovirus (influenza virus) resistance 1	NM_002462	MX1	1	0.796	610.251	258.706
Myeloid differentiation primary response gene (88)	NM_002468	MYD88	1	0.724	5.846	2.418
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_003998	NFKB1	1	0.625	2.663	0.656
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NM_020529	NFKBIA	1	7.085	8.874	27.758
2'-5'-oligoadenylate synthetase 2	NM_002535	OAS2	1	0.328	3702.438	466.499
Toll-like receptor 3	NM_003265	TLR3	1	0.391	47.242	2.865
Toll-like receptor 7	NM_016562	TLR7	1	0.658	4.483	1.126
Toll-like receptor 8	NM_138636	TLR8	1	-	1.762	4.227
Toll-like receptor 9	NM_017442	TLR9	1	0.677	1.539	2.966
Tumor necrosis factor	NM_000594	TNF	1	2.692	33.506	53.760
TNF receptor-associated factor 3	NM_003300	TRAF3	1	0.485	1.381	0.342
TNF receptor-associated factor 6	NM_004620	TRAF6	1	0.480	1.381	0.420

本實驗將載體細胞（A549 cell）分別加入 Emodin、加入流感病毒、同時加入 Emodin 和流感病毒作用 24 小時後，偵測經由第一型干擾素路徑（type 1 Interferon pathway）抗病毒基因之表現。結果 Interferon, betal 1、Interferon regulatory factor 7、ISG15 ubiquitin-like modifier、Myxovirus(influenza virus) resistance 1、2’-5’-oligoadenylate synthetase 2 在同時加入 Emodin 和流感病毒組有上升的情況。

表三、虎杖暨其化合物治療受感染的 A549 細胞後，對於經由第一型干擾素路徑的抗病毒基因之表現

Table 1. Gene expression levels of anti-virus replication related genes.

Gene Symbol	PBS	Virus	PC*	Virus/PC*	Emodin	Virus/Emodin	Resveratrol	Virus/Resveratrol
IFNA1	1±0.2	1.5±0.4	0.7±0.3	1.1±0.2	1.0±0.3	1.2±0.2	1.2±0.7	2.7±1.2
IFNB1	1±0.1	591.9±23.2$^{\#}$	0.3±0.1$^{\#,\&}$	992.8±123.1$^{\#,\&}$	0.3±0.1$^{\#,\&}$	492.3±86.1$^{\#}$	0.8±0.1$^{\#,\&}$	1093.7±98.1$^{\#,\&}$
IRF3	1±0.5	1.1±0.4	1.6±0.4	3.2±1.2$^{\#,\&}$	1.8±0.6	1.3±0.9	0.9±0.4	0.9±0.7
IRF5	1±0.2	0.8±0.3	2.5±0.9$^{\#,\&}$	4.0±0.9$^{\#,\&}$	2.1±0.6$^{\#,\&}$	1.4±0.1$^{\#,\&}$	2.3±0.2$^{\#,\&}$	2.9±0.9$^{\#,\&}$
IRF7	1±0.1	88.1±12.1$^{\#}$	4.0±1.1$^{\#,\&}$	633.0±45.2$^{\#,\&}$	4.5±1.6$^{\#,\&}$	134.2±18.2$^{\#,\&}$	3.9±0.8$^{\#,\&}$	127.6±13.3$^{\#,\&}$
ISG15	1±0.3	1126.3±90.1$^{\#}$	1.1±0.8$^{\&}$	2340.7±164.4$^{\#,\&}$	1.8±0.8$^{\&}$	501.8±78.2$^{\#,\&}$	2.1±0.9$^{\&}$	2462.7±154.2$^{\#,\&}$
MX1	1±0.4	766.3±60.5$^{\#}$	1.8±0.6$^{\&}$	1026.1±79.1$^{\#,\&}$	0.8±0.2$^{\&}$	258.7±34.1$^{\#,\&}$	3.6±0.9$^{\#,\&}$	1199.3±285.1$^{\#}$
MYD88	1±0.1	6.9±1.4$^{\#}$	1.8±0.4$^{\#,\&}$	26.1±5.6$^{\#,\&}$	0.7±0.4$^{\&}$	2.4±0.2$^{\#,\&}$	0.9±0.5$^{\&}$	6.3±1.2$^{\#,\&}$
NFKB1	1±0.2	2.8±0.98$^{\#}$	0.8±0.2$^{\&}$	1.5±0.3	0.6±0.3$^{\&}$	0.7±0.2$^{\&}$	1.1±0.9	2.4±3.2
NFKBIA	1±0.3	8.7±4.3$^{\#}$	2.4±0.6$^{\#}$	27.9±4.2$^{\#,\&}$	7.1±3.6$^{\#}$	27.8±4.9$^{\#,\&}$	1.7±1.1	6.8±1.4$^{\#}$
OAS2	1±0.2	2586. 0±123.1$^{\#}$	0.3±0.7$^{\&}$	2055.1±143.1$^{\#,\&}$	0.3±0.1$^{\#,\&}$	466.5±39.5$^{\#,\&}$	2.0±1.8$^{\&}$	3422.9±453.1$^{\#,\&}$
TLR3	1±0.4	42.6±14.3$^{\#}$	1.4±0.1$^{\&}$	46.7±9.8$^{\#}$	0.4±0.2$^{\&}$	2.9±0.5$^{\#,\&}$	2.6±1.2$^{\&}$	69.6±24.2$^{\#}$
TLR7	1±0.1	13.7±4.2$^{\#}$	0.9±0.3$^{\&}$	3.7±1.6$^{\&}$	0.7±0.1$^{\&}$	1.1±0.5$^{\&}$	0.1±0.1$^{\&}$	3.2±1.6$^{\&}$
TLR9	**1±0.1**	**1.5±0.3**	**2.5±0.3**$^{\#,\&}$	**3.0±0.8**$^{\#,\&}$	**0.7±0.7**	**3.0±0.2**$^{\#,\&}$	**5.5±0.4**$^{\#,\&}$	**5.5±0.1**$^{\#,\&}$
TNF	1±0.2	45.8±12.1$^{\#}$	1.2±0.3$^{\&}$	47.8±8.1$^{\#}$	2.7±0.7$^{\#,\&}$	53.8±10.1$^{\#}$	17.5±3.2$^{\#,\&}$	138.6±54.1$^{\#,\&}$
TRAF3	1±0.1	1.4±0.3	0.8±0.2$^{\&}$	1.2±0.4	0.5±0.1$^{\#,\&}$	0.3±0.4$^{\#,\&}$	1.3±0.4	1.6±0.8
TRAF6	1±0.3	1.2±0.7	0.9±0.7	1.0±0.4	0.5±0.2	0.4±0.8	1.2±0.2	1.6±0.7

*pc：虎杖。

表示 P 值 (配對比較控制組和病毒組或病毒組或藥物或病毒加藥物組)

小於 0.05。

& 表示 P 值 (配對比較病毒組和藥物，或病毒加藥物組) 小於 0.05。

結果的平均值 ± 標準差是一式三份進行的 3 個獨立實驗。

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歷年著作

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RESEARCH ARTICLE

Polygonum cuspidatum and Its Active Components Inhibit Replication of the Influenza Virus through Toll-Like Receptor 9-Induced Interferon Beta Expression

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Abstract

Influenza virus infection is a global public health issue. The effectiveness of antiviral therapies for influenza has been limited by the emergence of drug-resistant viral strains. Therefore, there is an urgent need to identify novel antiviral therapies. Here we tested the effects of 300 traditional Chinese medicines on the replication of various influenza virus strains in a lung cell line, A549, using an influenza-specific luciferase reporter assay. Of the traditional medicines tested, *Polygonum cuspidatum* (PC) and its active components, resveratrol and emodin, were found to attenuate influenza viral replication in A549 cells. Furthermore, they preferentially inhibited the replication of influenza A virus, including clinical strains isolated in 2009 and 2011 in Taiwan and the laboratory strain A/WSN/33 (H1N1). In addition to inhibiting the expression of hemagglutinin and neuraminidase, PC, emodin, and resveratrol also increased the expression of interferon beta (IFN-β) through Toll-like receptor 9 (TLR9). Moreover, the anti-viral activity of IFN-β or resveratrol was reduced when the A549 cells were treated with neutralizing anti-IFN-β antibodies or a TLR9 inhibitor, suggesting that IFN-β likely acts synergistically with resveratrol to inhibit H1N1 replication. This potential antiviral mechanism, involving direct inhibition of virus replication and simultaneous activation of the host immune response, has not been previously described for a single antiviral molecule. In conclusion, our data support the use of PC, resveratrol or emodin for inhibiting influenza virus replication directly and via TLR-9–induced IFN-β production.

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Introduction

The influenza virus is one of the most infectious respiratory tract pathogens and is annually responsible for significant levels of morbidity, mortality, and economic loss [1]. In recent years, influenza infection has garnered considerable attention as a public health issue. This is largely a result of a novel swine-origin H1N1 influenza virus that emerged in Mexico in April 2009 and rapidly spread worldwide [2]. The H1N1 influenza outbreak was the first pandemic of the twenty-first century. In March 2013, the highly pathogenic novel reassortant avian-origin influenza A virus, H7N9, emerged in Shanghai and Anhui, China. This virus caused rapidly progressing lower respiratory tract infections [3].

Currently, there are two classes of antiviral drugs approved by the US Food and Drug Administration (FDA) to treat or prevent influenza virus infections: M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) [4]. The use of M2 inhibitors is limited because of widespread drug resistance. Therefore, only neuraminidase inhibitors are widely used in the treatment of seasonal and pandemic influenza infections. However, oseltamivir-resistant viruses with the NA H275Y mutation were found to be widespread in seasonal H1N1 since 2007 and have circulated worldwide since the 2007–2008 influenza season [5,6]. The continuing threat of high spread efficiency and the potential emergence of more resistant strains necessitate the identification of new anti-influenza drugs to treat both seasonal and pandemic influenza.

One of the raw materials used for the production of oseltamivir is shikimic acid, which is derived from the traditional Chinese spice, star anise (*Illicium verum*) [7]. Medicinal plant extracts have been widely used in traditional medicine, and several plants have been investigated and shown to exhibit significant activity against the influenza virus [8,9], however, the molecular mechanisms of these herbs remain unclear and require further investigation.

Polygonum cuspidatum (PC), commonly called Japanese knotweed, is a member of the buckwheat family (Polygonaceae). Eight active compounds have been identified from PC extract: 2-methoxystypandrone (1); emodin (2); resveratrol (3); polydatin (4); emodin-8-O-beta-D-glucopyranoside (5); (E)-3,5, 12-trihydroxystilbene-3-O-beta-D-glucopyranoside-2'-(3",4",5"-trihydroxybenzoate) (6); and catechin-3-O-gallate (7); and rubiadin (8). PC has historically been used as a laxative, and occasionally as a food. Recent studies have reported that PC extract and its active components have antipyretic and analgesic activities [10–12].

Toll-like receptor 9 (TLR9) was first identified as the receptor for unmethylated cytosine-phosphate-guanine motifs in bacteria, and several TLR9 ligands have been identified in viruses and fungi [13,14]. TLR9 resides in endolysosomal compartments and is transduced via the adapter protein myeloid differentiation primary response gene (88) (MyD88), resulting in the activation of two different pathways.

The first pathway is a nuclear factor κB (NF-κB)-dependent pro-inflammatory cytokine pathway, and the second involves the activation of type I interferon (IFN) genes through IFN regulatory factor 7 (IRF7) phosphorylation [15,16]. IRF7 has been identified as a key regulator of IFN induction [17]. The type I IFN (alpha and beta IFN) response is one of the first lines of defense against viral infections. IFN-α/β were reported to exhibit antiviral activity when secreted by cells treated with heat-inactivated influenza A virus [18]. It has also been reported that influenza viruses are poor IFN-α/β inducers. This is because influenza viruses employ mechanisms to evade and antagonize the IFN-α/β response through the nonstructural protein 1 (NS1) gene [19].

In this study, we screened 300 antipyretic Chinese herbal medicines using a cell-based, high-throughput screening system. The active compounds identified during screening were also investigated for their mechanisms of action.

Materials and Methods

Cell culture and viruses

Madin-Darby canine kidney (MDCK) cells, human embryonal kidney (293T) cells and A549 lung cancer cells were obtained from the Bioresource Collection and Research Center (HsinChu, Taiwan) and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) and incubated in a 5% CO_2 humidified incubator. pREP4-FluA-Luc was transfected into 293T cells. Stable clones were selected with 250 μg/mL hygromycin B (Roche Diagnostics) and limiting dilution was performed to isolate clones with the highest luciferase activity when infected with influenza A virus. The influenza A/WSN/33 (H1N1) virus (WSN) was obtained from the American Type Culture Collection (Manassas, VA, USA).

Influenza A reporter construction

The influenza A reporter (pREP4-FluA-Luc) was constructed according to the method of Lutz et al. [20]. Briefly, luciferase gene was amplified by primers: lucfor: 5′-ATACGTCTCGGGG AGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTAAAATGGAAG ACGCCAAAAACATAAAF-3′; lucrev: 5′-ATACGTCTCATATTAGTAGAAACAAGGG TATTTTTCTTTACAATTTGGACTTTCCGCCC-3′. The amplified fragment was then cloned into the pHH21 plasmid (a generous gift from Prof. Earl Brown, University of Ottawa, Ottawa, Ontario, Canada). The DNA fragment containing RNA polymerase promoter I, the luciferase reporter, and RNA polymerase I terminator was cloned into pREP4 (Invitrogen).

Virus infection and assay procedure

A549 cells were seeded into 96-well plates (1×10^4 cells/well) and incubated at 37°C and 5% CO_2 for 24 h. The cells were then washed twice with phosphate buffered saline (PBS). Influenza A virus was diluted with PBS containing 0.2% bovine serum albumin to 10 multiplicity of infection (MOI)/100 μL. Cells were infected with 10 MOI H1N1 and centrifuged at 2000 rpm at room temperature for 1 h. Cells were washed twice with PBS and the test compounds were added. The 96-well plates were then incubated at 33°C and 5% CO_2 for 24 h. The supernatants containing the replicated virus were then added to 293T cells stably transfected pREP4-FluA-Luc and incubated at 37°C and 5% CO_2 for 24 h. Luciferase activity was determined by a luciferase assay system (Promega, Madison, WI, USA).

For the TLR9 inhibition assay, A549 cells were pre-treated with the TLR9 inhibitor, Super-iODN, (Enzo Life Sciences, Farmingdale, NY, USA) at 2 μM for 3 h before infection with the influenza virus. For IFN-β neutralization, anti-IFN-β antibodies (eBioscience, San Diego, CA, USA) were applied together with diluted influenza virus at a concentration of 10 μg/mL.

Quantitation of IFN-β

Cell culture supernatants were harvested 24 h after treatment and used directly for measurement of IFN-β concentration. IFN-β concentrations were determined using human IFN-β ELISA kit (PBL Assay Science, Piscataway, NJ, USA).

Western blotting

Western blotting was performed on A549 cells infected with H1N1 virus and treated with PC and its active components. Cells were harvested, washed with 10 mL of PBS three times, and lysed with mammalian protein extraction reagent (Thermo Fisher Scientific, Inc., Rockford, IL,

USA) containing a phosphatase inhibitor and a protease inhibitor. Proteins were separated on a 12.5% sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto a polyvinylidene difluoride membrane. The antibodies used to detect H1N1 hemagglutinin and neuraminidase were generated by immunization of Balb/C mice with H1N1 proteins (Fitzgerald Industries, Acton, MA, USA). For visualization, membranes were probed with an anti-mouse IgG secondary antibody conjugated to horseradish peroxidase. Binding was detected by a chemiluminescent substrate according to the manufacturer's instructions (Thermo Fisher Scientific). Blots shown are representative of at least three individual experiments.

Plaque reduction assay

MDCK cells were seeded into 6-well plates (1×10^6 cells/well). After incubation at 37°C and 5% CO_2 overnight, cells were washed twice with PBS and infected with H1N1 influenza virus (100 plaque forming units (PFU)/well) and kept on ice for 1 h. The cells were treated with PC or its active components in DMEM containing 0.3% agarose. The plate was incubated at 37°C and 5% CO2 for 72 h. The media were removed and the cells were fixed with 4% paraformaldehyde for 1 h at room temperature and subsequently stained with 0.1% crystal violet for 20 min at room temperature.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted by using an RNA isolation kit (Qiagen, Valencia, CA, USA), and 5 μg of RNA were reverse-transcribed using a reverse transcriptase for conversion to cDNA (Invitrogen). Primers and probes used for qPCR were selected from a predesigned probe system (Roche, Burgess Hill, UK). The RNA abundance was normalized to glyceraldehyde 3-phosphate dehydrogenase RNA in each sample.

Results

The antiviral activities of PC were evaluated using a virus-inducible reporter system. H1N1 virus (10 MOI) was used to infect A549 lung cancer cells for 1 h, and the cells were then treated with a water extract of PC. The A549 culture supernatant containing the amplified H1N1 was then applied to 293T cells stably transfected with influenza A reporter plasmid (pREP4-FluA-Luc). PC inhibited H1N1 replication in A549 cells as shown by reduced luciferase activity (Fig. 1A). The IC_{50} value for PC was 312 μg/mL.

Four active ingredients of PC (rubiadin, resveratrol, emodin, and polydatin) were also tested to determine which compound(s) exhibited inhibitory effects on H1N1 replication in A549 cells. The IC_{50} values for rubiadin, resveratrol, emodin, and polydatin were >50 μM, 24.7 μM, 37.3 μM, and >50 μM, respectively. These results indicate that resveratrol and emodin were the most potent active compounds from PC for inhibition of H1N1 viral replication. We also determined the cytotoxicity of PC, rubiadin, resveratrol, emodin, and polydatin on A549 cells. PC, rubiadin, resveratrol, emodin, and polydatin exhibited low toxicity against A549 cells, with GI_{50} values of >500 μg/mL for PC (Fig. 1C) and >100 μM for rubiadin, resveratrol, emodin, and polydatin (Fig. 1D).

One clinically isolated strain of H3N2 influenza virus (obtained in 2009) and 10 clinically isolated strains of H1N1 influenza viruses (collected in 2009 and 2011) were also treated with PC (Fig. 2A) and resveratrol (Fig. 2B). These compounds inhibited the replication of the clinical isolates with potencies similar to those observed for the laboratory strain of H1N1 influenza, A/WSN/33.

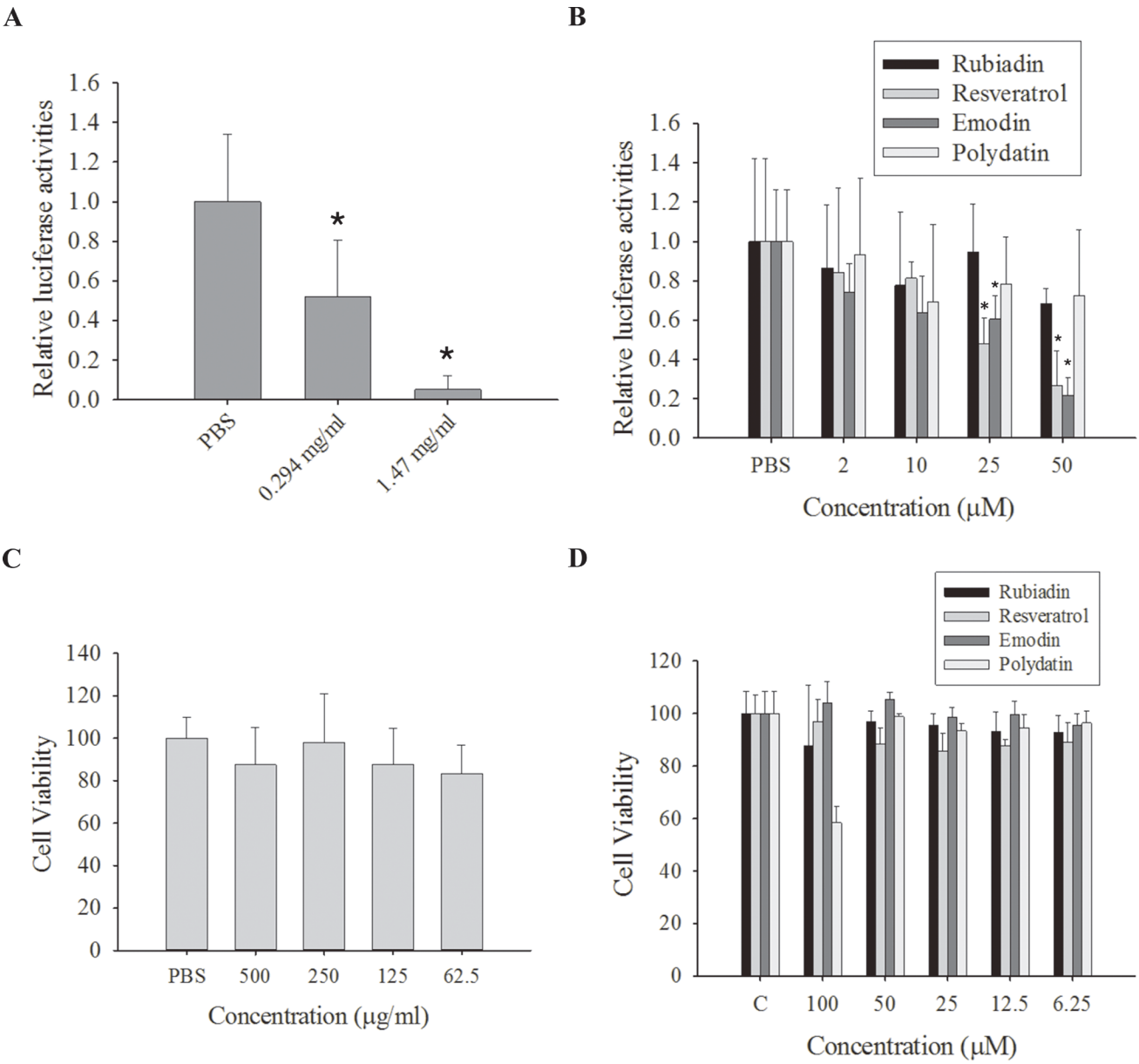

Fig 1. *Polygonum cuspidatum* and its active components inhibit replication of influenza A virus in A549 cells. (A) A water extract of *Polygonum cuspidatum* (PC) significantly inhibits influenza A virus replication in A549 cells. A549 cells were infected with 10 multiplicity of infection (MOI) H1N1 and treated with vehicle (phosphate buffered saline; PBS) or PC (0.294 and 1.47 mg/mL). The amplified virus in the culture supernatant was applied to 293T cells stably transfected with an influenza A reporter to determine influenza A replication in the presence of PC. (B) The active PC components rubiadin, resveratrol, emodin, and polydatin inhibited influenza A virus replication in A549 cells. A549 cells were infected with 10 MOI H1N1 treated with vehicle (PBS) or different concentration of rubiadin, resveratrol, emodin, and polydatin. The amplified virus in the culture supernatant was applied to 293T cells stably transfected with influenza A reporter to determine influenza A replication in the presence of rubiadin, resveratrol, emodin, and polydatin. Results are the mean ± standard deviation of three independent experiments performed in triplicate. Asterisks indicate the calculated p values for paired comparisons between control and drug treated samples are <0.05. (C and D) A549 cells were seeded into 96-well plates at a concentration of 5000 cells/well. Different concentrations of PC (C) or rubiadin, resveratrol, emodin, and polydatin (D) were added to each well. After 24 h, the cytotoxicity was determined by MTT assay. Results are the mean ± standard deviation of three independent experiments performed in triplicate.

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As shown in Fig. 3A and B, a plaque reduction assay was performed to confirm the antiviral activities of PC, resveratrol, and emodin. The plaque numbers were significantly reduced in response to 1.47 mg/mL PC (well 2), 0.294 mg/mL PC (well 5), 25 μM resveratrol (well 3), and

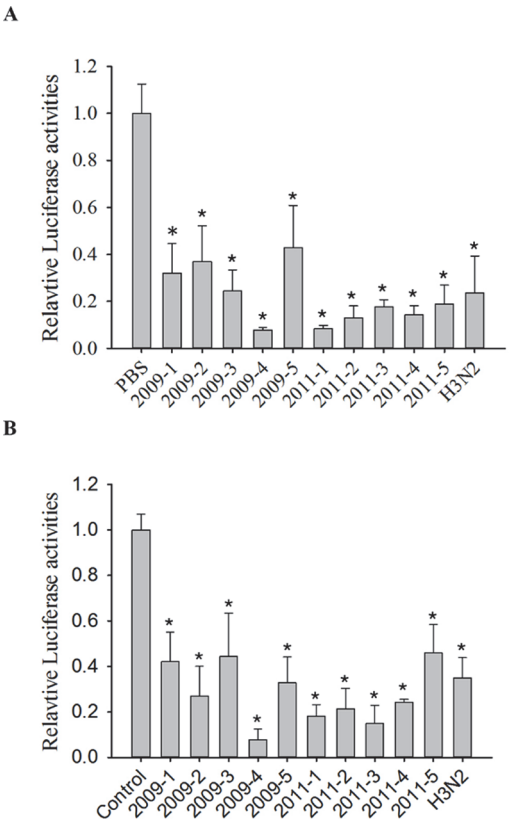

Fig 2. *Polygonum cuspidatum* (PC) and resveratrol inhibit the replication of H1N1 and H3N2 influenza A virus. (A) The water extract of PC significantly inhibited influenza A virus replication in A549 cells. A549 cells were infected with 10 multiplicity of infection (MOI) H1N1 treated with vehicle (phosphate buffered saline; PBS) or PC (0.3 mg/mL). The amplified virus in the culture supernatant was applied to 293T cells stably transfected with the influenza A reporter to determine influenza A replication in the presence of PC. (B) Resveratrol inhibits influenza A virus replication in A549 cells. A549 cells were infected with 10 MOI H1N1 treated with vehicle (PBS) or with 25 μM resveratrol. The amplified virus in the culture supernatant was applied to 293T cells stably transfected with influenza A reporter to determine influenza A replication in the presence of resveratrol. Results are the mean ± standard deviation of three independent experiments performed in triplicate. Asterisks indicate the calculated p values for paired comparisons between control and drug treated samples are <0.05.

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25 μM emodin (well 6). Emodin inhibited hemagglutinin expression (Fig. 3C; lane 5) while PC and resveratrol reduced hemagglutinin and neuraminidase expression (Fig. 3C; lanes 3 and 6; respectively). Treatment with PC and its active ingredients inhibited the expression of H1N1 hemagglutinin (Fig. 3D) and neuraminidase (Fig. 3E) in A549 cells infected with H1N1. Taken together, these data indicate that PC inhibited H1N1 replication in A549 cells.

To investigate the underlying mechanisms of PC, emodin, and resveratrol in inhibiting influenza virus replication, we performed real-time PCR analysis for several antiviral-related genes. A549 cells were treated with PBS (control), the compounds alone, the virus alone, or the compounds and the virus. We found that TLR9 mRNA expression was increased when the cells were treated with PC, emodin, or resveratrol alone or when they were infected with virus and then treated with PC (294 μg/mL), emodin (25 μM), or resveratrol (25 μM). In contrast, the TLR9 mRNA expression level was unchanged when the cells were infected with the virus alone (Fig. 4A and Table 1). TLR9 protein expression level increased in A549 cells treated with

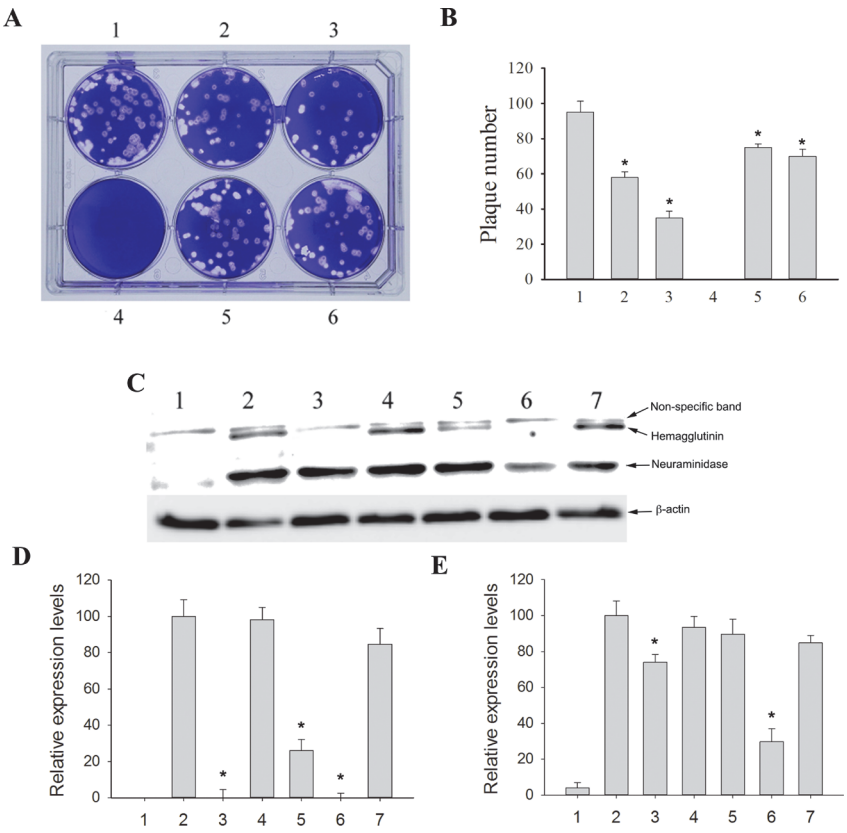

Fig 3. *Polygonum cuspidatum* (PC), resveratrol, and emodin reduced plaque numbers in a plaque reduction assay and inhibited the accumulation of hemagglutinin and neuraminidase in A549 cells. (A) *Polygonum cuspidatum* (PC), resveratrol, and emodin reduced plaque numbers in a plaque reduction assay. A representative plaque reduction assay is shown. Madin-Darby canine kidney (MDCK) cells were seeded into 6-well plates (1×10^6 cells/well) and infected with H1N1 influenza virus (100 PFU/well). The cells were treated with PC or its active components in Dulbecco's modified Eagle medium containing 0.3% agarose. After 72 h, the plaques were determined by staining with 0.1% crystal violet. Well 1: virus control; well 2: virus + PC (1.47 mg/mL); well 3: virus + 25 μM resveratrol; well 4: mock; well 5: virus + PC (0.294 mg/mL); well 6: virus + 25 μM emodin. (B) PC, resveratrol, and emodin reduced plaque numbers in a plaque reduction assay. Results are the mean ± standard deviation of three independent experiments performed in triplicate. Asterisks indicate the calculated p values for paired comparisons between 1 and 2, 3, 5, and 6 are <0.05. (C) PC and its active components inhibit the accumulation of hemagglutinin and neuraminidase in A549 cells. A549 cells were infected with H1N1 and treated with PC or its active components and the cell extracts were collected at 24 h post infection. Lane 1: mock; lane 2: virus only; lane 3: PC (1.47 mg/mL); lane 4: rubiadin (25 μM); lane 5: emodin (25 μM); lane 6: resveratrol (25 μM); lane 7: polydatin (25 μM). The result shown is one of the Western blots from three independent experiments. (D) Densitometry quantification of the hemagglutinin expression levels from the Western blot analyses. (E) Densitometry quantification of the neuraminidase expression levels from the Western blot analyses. Results are the mean ± standard deviation of three independent experiments performed in triplicate.

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resveratrol or resveratrol + H1N1, but was unchanged in cells infected with H1N1 alone (Fig. 4B).

The expression MYD88 mRNA was significantly increased with PC treatment. This is significant since MYD88 is involved in signal transduction resulting from TLR9 activation. It has been shown that TLR9 activates the IFN regulatory factors IRF3, IRF5, and IRF7 and the NF-κB family. IRF5 and IRF7 are strong transcriptional activators that lead to the expression of IFN-α and IFN-β. We found that PC, emodin, and resveratrol treatments significantly increased IRF5 and IRF7 mRNA expression (Table 1). The IRF7 mRNA levels were 7-fold higher when cells were treated with H1N1 + PC compared to A549 cells treated only with H1N1 ($p < 0.05$).

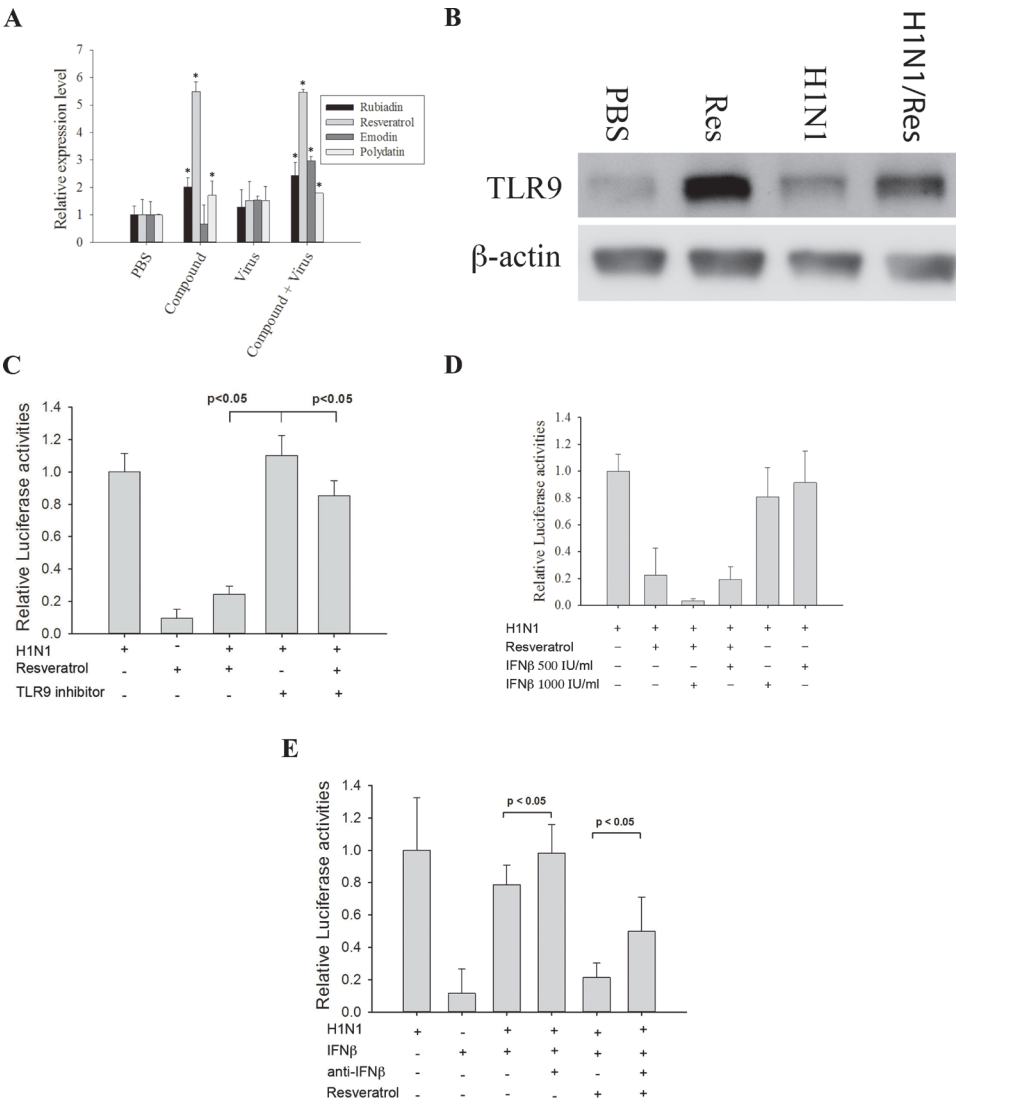

Fig 4. TLR9 and IFN-β acted synergistically with resveratrol to inhibit virus infection. (A) Resveratrol induces the expression of Toll-like receptor 9 (TLR9). A549 cells were treated as indicated and total RNAs were extracted to determine the TLR9 expression levels by real-time quantitative polymerase chain reaction (PCR). Results are the mean ± standard deviation of three independent experiments performed in triplicate. Asterisks indicate the calculated p values for paired comparisons between control (phosphate buffered saline; PBS) and drug treated samples are <0.05. (B) Resveratrol increased the expression level of TLR9. A549 cells were treated with PBS (control), 25 μM resveratrol, H1N1, H1N1 + 25 μM resveratrol and the cell extracts were collected at 24 h post infection. The result shown is one of the Western blots from three independent experiments. (C) Inhibiting TLR9 reduced the anti-H1N1 effect of resveratrol. A549 cells were treated with/without TLR9 inhibitor for 3 h before infected with H1N1. After 24 h of infection, the amplified virus in the culture supernatant was applied to 293T cells stably transfected with the influenza A reporter to determine influenza A replication in the presence of a TLR9 inhibitor. (D) Resveratrol works synergistically with interferon-β (IFN-β) to inhibit the replication of influenza A virus in lung cells. The treatments applied to A549 cells are listed in the figure. The amplified virus in the culture supernatant was applied to 293T cells stably transfected with influenza A reporter to determine influenza A replication in the presence of different treatments. The p values from Student's t-tests for the paired comparisons of 25 μM resveratrol; 25 μM resveratrol + 1000 IU/mL IFN-β; 25 μM resveratrol + 500 IU/mL IFN-β versus the control are all <0.05. The p value from Student's t-tests for the paired comparisons of 25 μM resveratrol versus 25 μM resveratrol + 1000 IU/mL IFN-β is <0.05. A p value < 0.05 is considered as significant. (E) Neutralizing IFN-β antibodies reduced the anti-viral activity of resveratrol. A549 cells were infected with 10 MOI H1N1. The treatments of A549 cells were listed in the figure. After 24 h of infection, the amplified virus in the culture supernatant was applied to 293T cells stably transfected with the influenza A reporter to determine influenza A replication in the presence of neutralizing anti-IFNβ antibodies. (F) A549 cells expressed IFN-β when treated with H1N1 or

H1N1 + resveratrol. The treatments applied to the A549 cells are listed in the figure. After 24 h of infection, cell culture supernatants were collected to determine the IFN-β concentration (by ELISA). The p value from Student's t-tests for the paired comparisons of H1N1 with H1N1 + resveratrol is <0.05. A p value <0.05 is considered as significant.

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We also investigated the expression of IFNα and IFN-β. As shown in Table 1, IFN-β mRNA levels were two-fold higher in H1N1 + PC and H1N1 + resveratrol treated cells than in A549 cells only infected with H1N1 ($p < 0.05$). We did not observe a significant increase in IFN-α mRNA expression in this study. Together, these results indicate that IFN-β plays an important role in the clearance of H1N1 infection when treated with PC, emodin, and resveratrol.

Secreted type I IFN induces the expression of myxovirus-resistance protein 1 (MX1), which is important in inhibiting influenza A replication. MX1 mRNA expression levels increased 1.3-fold when cells were treated with H1N1 + PC or resveratrol compared to cells only infected with H1N1 ($p < 0.05$). In addition, interferon-induced protein IFI-15k (ISG15) and 2′,5′-oligoadenylate synthetase (OAS2) mRNAs exhibited expression patterns that were similar to MX1 (Table 1).

A specific TLR9 inhibitor, Super-iODN, was used to evaluate the importance of TLR9 in inhibiting influenza virus replication. We performed a reporter assay using resveratrol alone or in combination with the TLR9 inhibitor. A549 cells were infected with 10 MOI H1N1 virus for 1 h and subsequently treated with resveratrol, the TLR9 inhibitor, or resveratrol + the TLR9 inhibitor. Inhibition of H1N1 replication by resveratrol was reduced when the cells were treated with the TLR9 inhibitor (Fig. 4C). This indicated the importance of TLR9 in resveratrol mediated inhibition of replication.

Table 1. Gene expression levels of anti-virus replication related genes.

Gene Symbol	PBS	Virus	PC*	Virus/PC*	Emodin	Virus/Emodin	Resveratrol	Virus/Resveratrol
IFNA1	1±0.2	1.5±0.4	0.7±0.3	1.1±0.2	1.0±0.3	1.2±0.2	1.2±0.7	2.7±1.2
IFNB1	1±0.1	591.9±23.2$^{\#}$	0.3±0.1$^{\#,\&}$	992.8±123.1$^{\#,\&}$	0.3±0.1$^{\#,\&}$	492.3±86.1$^{\#}$	0.8±0.1$^{\#,\&}$	1093.7±98.1$^{\#,\&}$
IRF3	1±0.5	1.1±0.4	1.6±0.4	3.2±1.2$^{\#,\&}$	1.8±0.6	1.3±0.9	0.9±0.4	0.9±0.7
IRF5	1±0.2	0.8±0.3	2.5±0.9$^{\#,\&}$	4.0±0.9$^{\#,\&}$	2.1±0.6$^{\#,\&}$	1.4±0.1$^{\#,\&}$	2.3±0.2$^{\#,\&}$	2.9±0.9$^{\#,\&}$
IRF7	1±0.1	88.1±12.1$^{\#}$	4.0±1.1$^{\#,\&}$	633.0±45.2$^{\#,\&}$	4.5±1.6$^{\#,\&}$	134.2±18.2$^{\#,\&}$	3.9±0.8$^{\#,\&}$	127.6±13.3$^{\#,\&}$
ISG15	1±0.3	1126.3±90.1$^{\#}$	1.1±0.8$^{\&}$	2340.7±164.4$^{\#,\&}$	1.8±0.8$^{\&}$	501.8±78.2$^{\#,\&}$	2.1±0.9$^{\&}$	2462.7±154.2$^{\#,\&}$
MX1	1±0.4	766.3±60.5$^{\#}$	1.8±0.6$^{\&}$	1026.1±79.1$^{\#,\&}$	0.8±0.2$^{\&}$	258.7±34.1$^{\#,\&}$	3.6±0.9$^{\#,\&}$	1199.3±285.1$^{\#}$
MYD88	1±0.1	6.9±1.4$^{\#}$	1.8±0.4$^{\#,\&}$	26.1±5.6$^{\#,\&}$	0.7±0.4$^{\&}$	2.4±0.2$^{\#,\&}$	0.9±0.5$^{\&}$	6.3±1.2$^{\#,\&}$
NFKB1	1±0.2	2.8±0.98$^{\#}$	0.8±0.2$^{\&}$	1.5±0.3	0.6±0.3$^{\&}$	0.7±0.2$^{\&}$	1.1±0.9	2.4±3.2
NFKBIA	1±0.3	8.7±4.3$^{\#}$	2.4±0.6$^{\#}$	27.9±4.2$^{\#,\&}$	7.1±3.6$^{\#}$	27.8±4.9$^{\#,\&}$	1.7±1.1	6.8±1.4$^{\#}$
OAS2	1±0.2	2586. 0±123.1$^{\#}$	0.3±0.7$^{\&}$	2055.1±143.1$^{\#,\&}$	0.3±0.1$^{\#,\&}$	466.5±39.5$^{\#,\&}$	2.0±1.8$^{\&}$	3422.9±453.1$^{\#,\&}$
TLR3	1±0.4	42.6±14.3$^{\#}$	1.4±0.1$^{\&}$	46.7±9.8$^{\#}$	0.4±0.2$^{\&}$	2.9±0.5$^{\#,\&}$	2.6±1.2$^{\&}$	69.6±24.2$^{\#}$
TLR7	1±0.1	13.7±4.2$^{\#}$	0.9±0.3$^{\&}$	3.7±1.6$^{\&}$	0.7±0.1$^{\&}$	1.1±0.5$^{\&}$	0.1±0.1$^{\&}$	3.2±1.6$^{\&}$
TLR9	**1±0.1**	**1.5±0.3**	**2.5±0.3**$^{\#,\&}$	**3.0±0.8**$^{\#,\&}$	**0.7±0.7**	**3.0±0.2**$^{\#,\&}$	**5.5±0.4**$^{\#,\&}$	**5.5±0.1**$^{\#,\&}$
TNF	1±0.2	45.8±12.1$^{\#}$	1.2±0.3$^{\&}$	47.8±8.1$^{\#}$	2.7±0.7$^{\#,\&}$	53.8±10.1$^{\#}$	17.5±3.2$^{\#,\&}$	138.6±54.1$^{\#,\&}$
TRAF3	1±0.1	1.4±0.3	0.8±0.2$^{\&}$	1.2±0.4	0.5±0.1$^{\#,\&}$	0.3±0.4$^{\#,\&}$	1.3±0.4	1.6±0.8
TRAF6	1±0.3	1.2±0.7	0.9±0.7	1.0±0.4	0.5±0.2	0.4±0.8	1.2±0.2	1.6±0.7

*PC: *Polygonum cuspidatum*

#indicate the calculated p values for paired comparisons between control and virus or drug or virus/drug treated samples are <0.05.

& indicate the calculated p values for paired comparisons between virus and drug or virus/drug treated samples are <0.05.

Results are the mean ± standard deviation of three independent experiments performed in triplicate.

doi:10.1371/journal.pone.0117602.t001

To determine the effect of type I IFN on the inhibition of H1N1 replication, we performed a reporter assay using IFN-β alone or in combination with resveratrol. A549 cells were infected with 10 MOI H1N1 virus for 1 h and treated with resveratrol, IFN-β, or IFN-β + resveratrol. Our results indicate that IFN-β alone slightly inhibited viral replication in A549 cells (Fig. 4D). IFN-β combined with resveratrol produced more significant inhibition of H1N1 infection than resveratrol alone. The inhibition of H1N1 replication by IFN-β or IFN-β + resveratrol was reduced when cells were treated with neutralizing antibody to IFN-β (Fig. 4E).

The expression levels of IFN-β were also determined after A549 cells were treated with resveratrol. A549 cells were infected with 10 MOI H1N1 virus and treated with resveratrol, TLR9 inhibitor or TLR9 inhibitor + resveratrol. The expression level of IFN-β was significantly higher in cells treated with resveratrol compared with cells treated with TLR9 inhibitor or TLR9 inhibitor + resveratrol (Fig. 4F). These results indicate that resveratrol worked synergistically with IFN-β to inhibit H1N1 infection in A549 lung cells *in vitro*.

Discussion

The aim of this study was to identify potential new treatments for influenza from traditional Chinese herbal medicines and their active components. We created an influenza A virus reporter cell system to screen potential candidates for inhibition of influenza virus replication. In addition, through investigation of the molecular mechanisms underlying these potential new treatments for influenza, we can assess potential new anti-virus pathways for developing more efficacious treatments.

In this study, we infected A549 cells with wild type influenza virus and treated them with various compounds. If a compound was effective in mitigating viral replication, viral RNA polymerase was present in lower amounts, leading to a concentration-dependent reduction in luciferase reporter levels. This indicated a concentration-dependent reduction in influenza virus replication.

Importantly, this screening process could be completed within 24 h instead of the 2–3 days necessary for plaque formation assays. These results indicated that our system was an effective method to assess influenza A viral titration. Notably, our reporter cell-based screening assay, as with similar assays, showed some level of toxicity to the cells by the test compounds, which could potentially lead to false positives. To avoid false positives, we performed MTT tests to exclude compounds that were toxic to the carrier cells.

In our small-scale screening program, we excluded toxic compounds and found that PC, emodin, and resveratrol exhibited significant inhibitory activity against H1N1 and H3N2 influenza A viruses with no significant toxic effect on the host cells. PC, resveratrol, and emodin exhibited potent antiviral activity against multiple subtypes of the influenza A virus, including the A/WSN/33 (H1N1) laboratory strain and the H3N2 and H1N1 clinical strains. Both of these clinically isolated strains have been shown to cause severe human respiratory disease. These strains were utilized in this study since both H3N2 and H1N1 have emerged during the twentieth century and caused four influenza A pandemics [21].

Hemagglutinin is responsible for the binding of the virus to the target cells to initiate viral infection, whereas neuraminidase cleaves the host cellular receptors and facilitates the release of the progeny virus, thus promoting the spread of the infection to neighboring cells [22,23]. Our western blots demonstrated that PC and two of its active components, emodin and resveratrol, reduced hemagglutinin and neuraminidase expression, indicating that they are capable of inhibiting viral replication.

The Type I IFN system (IFN-α/β) is the first line of defense against viral infections [24,25]. These molecules act directly on virus-infected cells, resulting in apoptosis, cytokine signaling, regulation of cell growth, and hematopoietic development [26]. PC, resveratrol, and emodin

induced only IFN-β in the presence of influenza virus infection. Since IFN-β is likely to induce a specific antiviral immune response only in infected cells, this limits the likelihood that the undesirable side effects associated with systemic IFN therapy would be produced. Furthermore, since PC, resveratrol, and emodin induced IFN-β while retaining their direct antiviral activity, these synergistic effects may be a simple and efficient way to reduce influenza viral yield. This also suggests that the antiviral mechanisms of the compounds may be dependent on type I IFN. Therefore, these data indicate that the activation of an innate immune response was secondary to a more direct antiviral function of these compounds.

The viral nonstructural protein 1 (NS1) of the influenza A virus prevents the induction of the IFN-β promoter by inhibiting the activation of transcription factors such as IRF3 [27]. Both IRF3 translocation and NF-κB activation are impaired in the presence of NS1, which blocks the induction of proinflammatory cytokines and IFNs [28,29]. Our results show that IRF3 and NF-κB were not induced, indicating that the antiviral mechanisms of PC, resveratrol, and emodin may not involve the NS1 protein.

TLR9 is involved in cellular antiviral mechanisms. TLR9 activates two distinct pathways: the NF-κB-dependent proinflammatory cytokine pathway and the IRF7-dependent type I IFN pathway [19,30,31]. IRF7 directly interacts with the MYD88 signaling adaptor, and engagement of TLR9 by non-replicating viral genomic content leads to the rapid secretion of IFNs. Within the IRF family, IRF3 and IRF7 have been identified as key regulators of the IFN induction. However, IRF7 is largely responsible for IFN production in response to influenza A infection, as shown by the abrogation of IFN production in IRF7$^{-/-}$ mice [32]. Our results indicate that the suppression of influenza virus replication and enhancement of IFN-β gene expression induced by PC, resveratrol, and emodin might be mediated through the regulation of IRF7.

In this study, we detected significantly elevated levels of TLR9 and reduced influenza virus A replication in cells treated with PC, resveratrol, or emodin compared to control cells ($P < 0.05$). This suggests that enhanced TLR9 production and the activation of virus-induced IRF7 induced IFN-β protein expression. Taken together, these data demonstrate that PC, resveratrol, or emodin, in the presence of the influenza A virus, result in the activation of IFN-β through the TLR9-MYD88–IRF7 pathway (Fig. 5).

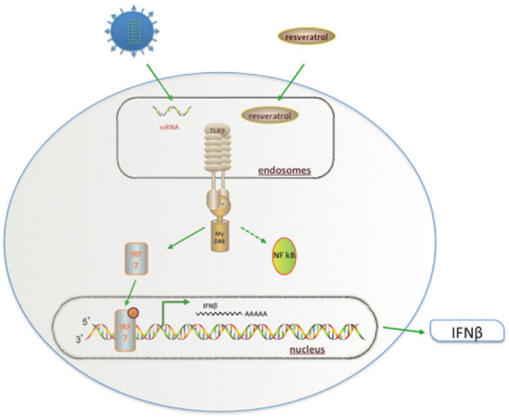

Fig 5. The molecular mechanisms underlying the antiviral activity of *Polygonum cuspidatum* (PC) and its active components. PC, resveratrol, and emodin enter the influenza-infected cell and then activate proinflammatory cytokines through TLR9. TLR9 signals through the adapter protein myeloid differentiation primary response gene 88 (MYD88), leading to the activation of two different pathways. PC, resveratrol, and emodin likely activate interferon regulator factor 7 (IRF7) pathway rather than the nuclear factor κB (NF-κB) pathway, leading to the activation of type I IFN genes through the phosphorylation of IRF7 and the transcriptional activation of proinflammatory cytokines.

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In this study, we identified anti-influenza A virus compounds by screening 300 Chinese herbal medicines in a luciferase reporter assay and found that the Chinese herbal compound, PC, and two of its active components, resveratrol and emodin, induced IFN-β only in the presence of influenza virus infection. Moreover, these three compounds exhibited a superior ability to induce the expression of IFN-β and ISGs. PC, resveratrol, and emodin inhibited the growth of influenza A viruses, including the clinically isolated strains H3N2 and H1N1 and the A/WSN/33 (H1N1) laboratory strain. We found that the antiviral activities of PC, resveratrol, and emodin were dependent on IFN production and signaling. This inhibitory effect was associated with the induction of IFN gene expression through the TLR9-MYD88–IRF7 pathway. Together, the findings of this study suggest that synergistic effects of antiviral compounds and IFN-β can protect against influenza virus infections. These insights may lead to the development of novel antiviral therapies for the treatment of influenza.

Author Contributions

Conceived and designed the experiments: CJL HJL THC YAH CSL GYH LW. Performed the experiments: CJL HJL. Analyzed the data: CSL THC. Contributed reagents/materials/analysis tools: LW GTH. Wrote the paper: CSL THC LW.

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